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13. ABSTRACT *(Maximum 200 words)*

Apoptosis is a biological process for cell removal, for which the underlying mechanism is not well understood. Restoring the pathways that control apoptosis holds promise as a new approach for the treatment of breast cancer. The objective of the research project is to test the hypothesis that oxidative stress plays a critical role in the mechanism of apoptosis induced by treatment of human breast cancer cell lines with tumor necrosis factor-α (TNF-α). The goal of the first year of the project was to use Northern blot hybridization analyses and enzyme assays to define the extent of changes in the cellular antioxidant defense in TNF-α-treated breast cancer cells. Our first studies demonstrated dose- and time-dependent increases in apoptosis, based on an ELISA for DNA fragments, with TNF-α treatment of MCF-7 cells. In conducting Northern blot hybridization analysis, we encountered problems that appeared to be due to UV damage to the cDNA probe templates, that occurred while purifying them. The problem was resolved by shielding the sample during the workup. For biochemical measurements of the antioxidant defense, we have a catalase assay underway and are comparing methods for measuring GSH levels.

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INTRODUCTION

Apoptosis, or programmed cell death, is a normal process used by the body to eliminate cells for the benefit of the organism. The aberrant growth of some cancer cells appears due in part to defects in the signalling or metabolic pathways that are essential for apoptosis. Restoring the pathways that control apoptosis holds promise as a new approach for the treatment of cancer. Strategies for the rational development of apoptosis-based breast cancer treatment are limited as long as the underlying, molecular mechanism of apoptosis in breast tissue remains poorly understood. Studies of a classical system of apoptosis: steroid hormone-induced lymphocyte apoptosis, have led us to the **hypothesis** that oxidative stress plays a critical role in the mechanism of apoptosis. The **objective** of the research project is to test this hypothesis using a human breast cancer cell model, in which apoptosis is induced by treatment of the cells with tumor necrosis factor-α (TNF-α).

The proposed **experiments** include: **1)** using Northern blot hybridization analyses and enzyme assays to define the extent of changes in the cellular antioxidant defense, following treatment of breast cancer cells with TNF-α; **2)** determining whether breast cancer cells that have been transfected with expression vectors encoding antioxidant defense genes are resistant to TNF-α-induced apoptosis; **3)** determining whether the induction of anti-sense antioxidant defense genes is sufficient to cause apoptosis of breast cancer cells, and **4)** investigating the effects of oxidative stress, in breast cancer cells, on redox-sensitive transcription factors. *The goal of the first year of the project was to complete Specific Aim #1.*

The proposed project is **significant** to the treatment of breast cancer because: **1)** it is likely that defects in the apoptotic pathway contribute to the conversion of normal breast tissue to malignant cancers; **2)** induction or restoration of apoptosis has promise as a novel approach to treating breast cancer; **3)** an understanding of the underlying molecular mechanism is needed to guide the rational development of apoptosis-based breast cancer treatments, and **4)** the proposed experiments test a specific hypothesis relevant to the mechanism of apoptosis in breast cancer cells.

1. Nature of the Problem

The concept of apoptosis was introduced in 1972 by Kerr, Wyllie and Currie, who presented arguments for this normal, biological process being an important mode of cell death in the body (1). Also referred to as programmed cell death, apoptosis functions during development to remove cells that are no longer needed (e.g., during tissue remodeling), or potentially harmful (e.g., nonfunctional, or self-reactive immune cells) (1,2). In adult tissues, apoptosis counterbalances mitosis and thus ensures homeostasis. In response to an appropriate stimulus, cells targeted for apoptosis condense and break down into membrane-enclosed fragments that are phagocytized by other cells. In this way, the cells "die" without evoking an inflammatory response and potentially harming neighboring, surviving cells.

As was suggested by Kerr *et al.*, hyperplasia in some cases may result from an inability of cells to die normally (1). Restoring the pathways that control apoptosis thus holds promise as a new approach for the treatment of cancer (3-5). The idea that defects in signalling or metabolic pathways essential to apoptosis contribute to the aberrant growth of cancer cells is supported by studies demonstrating that inappropriate expression of an oncogene, *bcl-2*, prevents cell death and thereby promotes

inappropriate survival of leukemic cells (6).

The current obstacle to developing strategies that use apoptosis to treat cancer is our lack of knowledge of the molecular mechanism at play and of the critical cellular components involved. The experiments proposed here address this lack of knowledge by testing a specific hypothesis relevant to the mechanism of TNF-α-induced apoptosis of breast cancer cells. Results from studies of a classic system of apoptosis: glucocorticoid treatment of lymphocytes, have led us to the hypothesis that oxidative stress plays a critical role in the mechanism of apoptosis. This hypothesis is supported by studies of apoptosis in other systems; in particular, TNF-α treatment of human breast cancer cells.

2. Background of previous work: Evidence for Oxidative Stress During Glucocorticoid-Induced Apoptosis

For many systems of apoptosis it appears that a genetic program is involved (7-10), but the questions of which genes are essential for the program, and the sequence and manner in which their products play a role remain largely unanswered. Systems of steroid hormone-mediated apoptosis are models of genetically-driven apoptosis since they are mediated by intracellular receptor proteins that function as steroid-activated transcription factors (11). Accordingly, steroid hormone-induced apoptosis has been found to be an active process that results in altered gene expression and requires ongoing protein synthesis (8-10,12). Identifying the hormone-responsive genes that are essential for apoptosis is an important first step toward a better understanding of programmed cell death. The goal of my postdoctoral studies was to use subtraction hybridization to clone genes that are differentially regulated during steroid hormone-mediated apoptosis and potentially critical to the mechanism of cell death (12). Subtraction hybridization allows for the direct isolation of transcripts that are differentially expressed. Once these transcripts are cloned, gene expression methods can be used to determine whether the respective genes are essential to apoptosis and a biochemical approach can be used to establish the role of the products of essential genes in the cell death pathway.

Before setting out to isolate transcripts that are differentially regulated during apoptosis, the feasibility of the task was assessed. Two-dimensional gel electrophoresis analyses of apoptosis induced in the rat ventral prostate (RVP) by androgen withdrawal or in thymocytes by glucocorticoid treatment demonstrate changes in a small number of proteins (13,14). This suggests that relatively few genes are differentially regulated and responsible for apoptosis in these systems. This suggestion was tested by conducting transcriptional analyses of glucocorticoid-treated hepatoma (HTC) and mouse lymphocyte (WEHI7.2) cell lines and of RVP tissue from normal *vs.* 3-day castrate rats. When ^{32}P-labeled cDNA prepared from HTC or WEHI7.2 cells incubated with dexamethasone (a potent, synthetic glucocorticoid) was hybridized with an excess of mRNA from untreated cells, only a fraction of the cDNA was left unhybridized (12). A greater percentage of unhybridized cDNA was seen following subtraction hybridization of RVP samples; however, this was largely due to one transcript, sulfated glycoprotien (SGP-2), making up greater than 70% of the unhybridized material. This suggests that a relatively small set of genes controls the process of apoptosis.

The results of the transcriptional analyses suggested to us that subtraction hybridization techniques were feasible for cloning transcripts that are differentially

regulated during apoptosis. Of the examples of apoptosis involving genetic control, regression of the RVP had an advantage in subtractive hybridization-based methods: a differentially-expressed transcript, SGP-2, had already been identified and cloned and could be used as an internal control during the cloning steps. With the construction and screening of an RVP subtraction cDNA library, 110 clones representing differentially-expressed genes were identified (15). After cross-hybridizations, Northern blot hybridization analyses and sequencing, four unique (non-SGP-2), differentially regulated genes were identified (Fig. 1). These genes encode the glutathione S-transferase Yb_1 subunit (GST), matrix carboxyglutamic acid protein, γ-actin and a novel sequence, which we designated RVP.1.

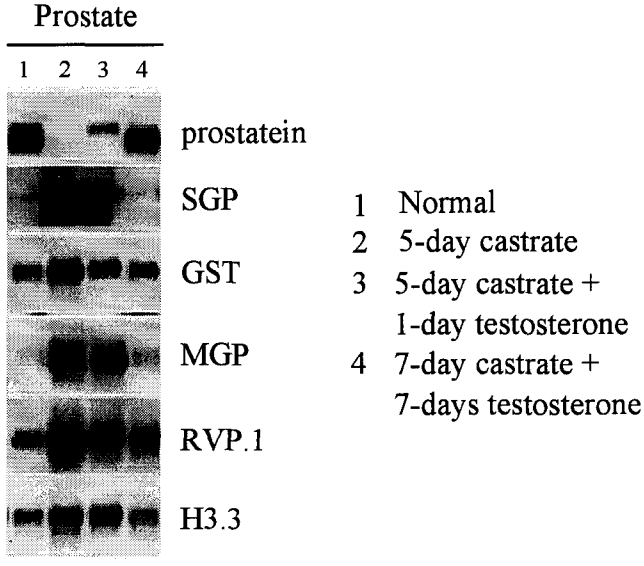


Figure 1. Identification of genes that are differentially regulated during apoptosis in the RVP. Total RNA was prepared from the RVP of rats treated as indicated in the Figure legend. Each lane was loaded with 20 µg RNA. Blots were probed with cloned sequences as indicated. Prostatein is the major secretory product of the RVP and is shown for comparison. The histone probe (H3.3) was used for standardization.

A disadvantage of the RVP for studies of apoptosis is that it is an *in vivo* model, and therefore not amenable to the genetic and biochemical studies necessary to determine whether GST, MGP, γ-actin or RVP.1 are critical for apoptosis. Therefore, we next tested whether these genes are differentially regulated in an *in vitro* model, glucocorticoid treatment of WEHI7.2 cells. Using Northern blot hybridization analysis, only GST was found to be differentially expressed during hormone-mediated apoptosis in this system (16). An increase in GST transcript levels was seen within 4 h of the addition of 1 µM dexamethasone to the cultures and after 24 h of treatment, GST mRNA levels were 12-fold higher in treated, *vs.* untreated cells (Fig. 2). We conclude that GST up-regulation is an early response, since it occurs well before a change in cell viability in the treated cultures, which occurs only after 24 h (data not shown).

GSTs are ubiquitous proteins that are part of the cell's antioxidant defense. This defense protects cells from the highly reactive oxygen radicals formed when electrons leak from the mitochondrial respiratory chain and other electron transfer systems and are captured by oxygen (17,18). Primary antioxidant defense genes (catalase, superoxide dismutases and DT-diaphorase) function to intercept reactive oxygen species before they can damage cellular targets (17). Thioredoxin, GSTs and DNA repair enzymes maintain a secondary line of defense by reversing oxidative damage (17,19). Endogenous compounds that are substrates for GSTs include lipids and DNA that have been damaged by oxidative stress (19).

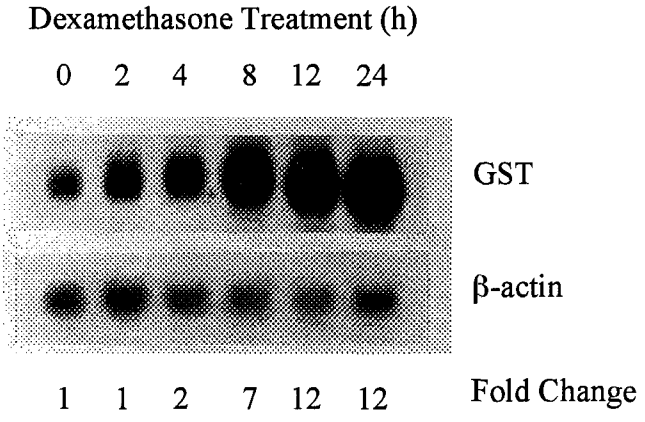
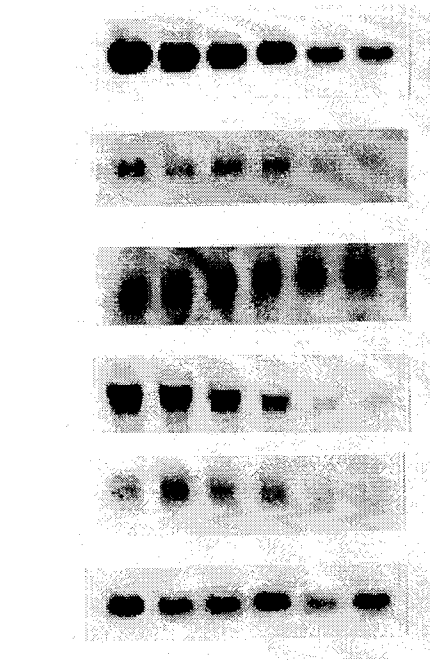


Figure 2. Upregulation of GST in dexamethasone-treated WEHI7.2 cells. Northern blot hybridization analysis of total RNA extracted from cells incubated with 1 μM dexamethasone for 0-24 h. The blot was hybridized with rat GST Yb_1 cDNA probe sequences, then stripped and rehybridized to mouse ß-actin probe sequences. From ref. 16.

Given its protective role in the cell, the increased expression of a GST subunit during apoptosis could be a response to oxidative stress. Subsequent experiments addressed the question of whether other antioxidant defense genes were differentially regulated during apoptosis. These experiments demonstrated that glucocorticoid treatment of WEHI7.2 cells results in the **downregulation** of the antioxidant defense genes encoding catalase, DT-diaphorase, manganese superoxide dismutase (MnSOD), copper,zinc-superoxide dismutase, (Cu,Zn-SOD) and thioredoxin (Fig. 3) (20). Again, the response appeared early, with changes occurring within 8 h of dexamethasone treatment. By 24 h, DT-diaphorase levels had decreased to 10% of the control value, catalase and thioredoxin to 20%, MnSOD to 30% and Cu,Zn-SOD to 50%. It should be re-emphasized that the products of these genes function to intercept oxygen radicals before their attack on cellular targets, while GSTs function to repair oxidative damage.

Multiple GSTs have been identified and are divided into three classes, based on biochemical and genetic criteria (21). Rat GST Yb_1 is a mu class subunit corresponding to murine mu isoenzymes, as described by Townsend *et al.* (22). Additional experiments addressed the question of whether up-regulation of GST gene expression during apoptosis was followed by an increase in protein levels, and whether other GST class isoenzymes were also differentially regulated. Western blot hybridization analysis indicated an increase in GST mu protein levels closely followed the change seen in message levels (Fig. 4). No differential expression was seen for GST Pi class isoenzyme (Fig. 4) and GST alpha subunits were not detected (data not shown).

Based on the results of the Western blot analysis, we expected an increase in GST enzyme activity with apoptosis. Increased GST activity could, furthermore, deplete cellular levels of the major intracellular redox buffer, reduced glutathione (GSH). Assays for GST activity and GSH in WEHI7.2 cell lysates prepared following dexamethasone treatment bore out these predictions (20). A significant increase in GST activity was seen after 12 h of dexamethasone treatment and at 24 h a 2-fold increase over the levels in untreated cells was measured. A decrease in GSH levels, to 80% of the control value, was observed after 24 h of dexamethasone treatment. While this change in GSH is probably not in itself lethal, it may enhance oxidative damage in light of the observed general decline in the expression of antioxidant defense genes. Additionally, depletion of GSH in localized areas of the cell could be significant to redox-sensitive proteins (23).



B Fold Change with Dex Treatment (h)

	0	2	4	8	12	24
Catalase	1.0	0.9	0.6	0.5	0.3	0.2
MnSOD	1.0	0.8	0.8	1.0	0.4	0.3
Cu,ZnSOD	1.0	0.6	0.8	0.6	0.4	0.5
Diaphorase	1.0	0.7	0.7	0.4	0.2	0.1
Thioredoxin	1.0	1.0	1.0	0.7	0.4	0.2
β-actin	1.0	1.0	1.0	1.0	1.0	1.0

Figure 3. Expression of antioxidant defense genes during apoptosis. A. Northern blot hybridization analyses of total RNA extracted from WEHI7.2 cells after incubation with 1 μM dexamethasone for 0-24 h. Blots were probed with cDNAs to antioxidant gene cDNAs, then stripped and probed with mouse ß-actin cDNA sequences. B. Quantitation of changes in gene expression with dexamethasone treatment. Values are relative to the untreated sample and normalized to the ß-actin results.

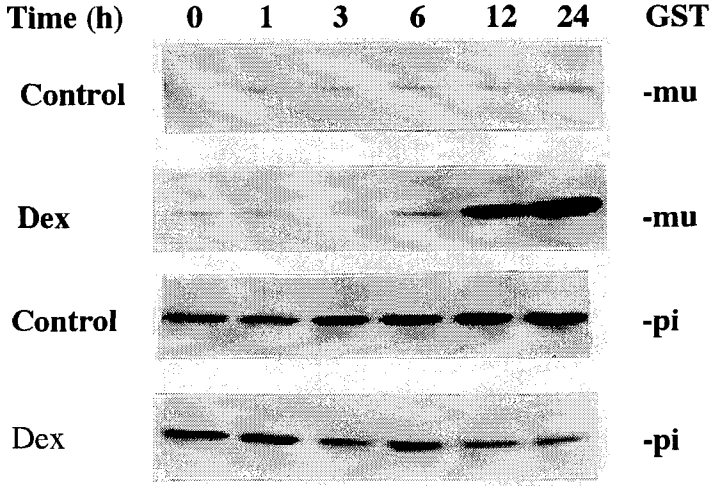


Figure 4. Western blot hybridization analysis of GST levels in dexamethasone-treated WEHI7.2 cells. Lysates prepared from control cells treated with vehicle alone (0.01% ethanol) or with 1 μM Dex for the indicated amounts of time were electrophoresed through 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose. GST mu and pi isoenzymes were detected with anti-rat GST Yb_1 and anti-human GST π antibodies, respectively, by indirect immunostaining with horseradish peroxidase. From ref. 20.

3. Evidence for the Role of Oxidative Stress in Other Systems of Apoptosis, Including Breast Cancer Cells

Having demonstrated the differential regulation of antioxidant defense genes and a lowering of GSH levels during apoptosis, *the next question to address is whether oxidative stress is critical to the underlying molecular mechanism of cell killing.* Results from several different groups support the hypothesis that oxidative stress plays a role in apoptosis. First, some of the cellular changes which occur during apoptosis are also observed with oxidative stress. Cellular changes seen during apoptosis include membrane blebbing and an increase in intracellular calcium (24,25). Orrenius and his colleagues have documented these same features in hepatocytes during oxidative stress induced by glutathione depletion (26). Second, Pierce *et al.* have found that the reactive oxygen species, hydrogen peroxide, mediates apoptosis in the blastocyst (27). In this case, the oxidative stress appears to be generated by activation of a polyamine oxidase (28). Third, defects in Cu,Zn-SOD and control of oxygen radicals have been linked to motor neuron death that occurs with amyotrophic lateral sclerosis (29-31).

Apoptosis of breast cancer cells is induced by the protein toxin, TNF-α (32). TNF-α is a 17.5 kDa protein, produced by activated macrophages and monocytes, which was isolated following the observation of tumor regression in cancer patients who contracted bacterial infections (reviewed in 33,34). Once cloned, recombinant, human TNF-α was found to exhibit cytotoxic effects on cells *in vitro* (35). While the mechanism of TNF-α-induced apoptosis is not known, oxidative stress appears to be involved. TNF-α cytotoxicity requires oxygen and is inhibited by free radical scavengers (36). Furthermore, the sensitivity of cells in cultures, to TNF-α, has been modulated by transfection of sense and anti-sense sequences of the antioxidant defense gene, MnSOD (37,38).

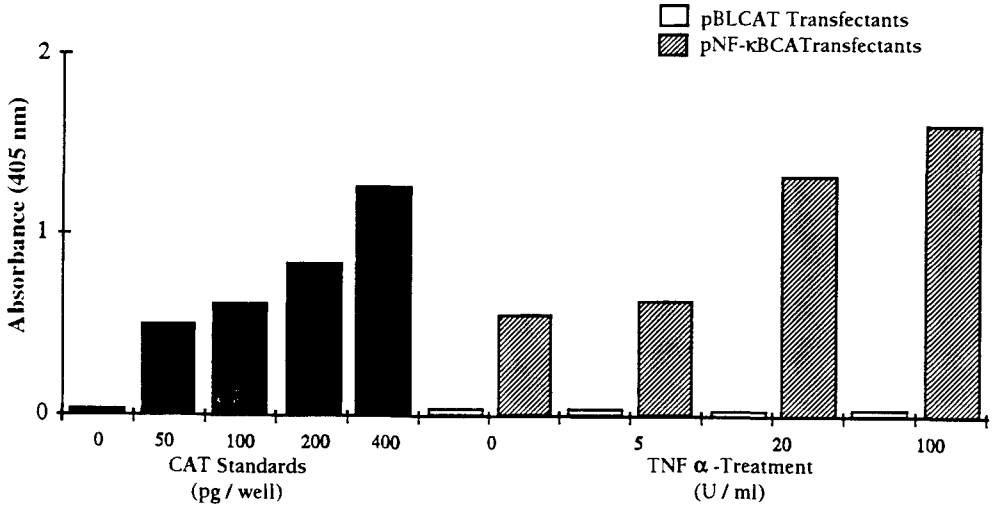


Figure 5. Activation of NF-κB in MCF-7 cells treated with TNF-α. The pNF-κBCAT reporter plasmid or pBLCAT control plasmid were introduced into MCF-7 cells by calcium phosphate precipitation. After a 24 h recovery period, cells were treated with TNF-α at the indicated concentrations. CAT-ELISA assays were performed after an additional 36 h. Results are the average of triplicate samples.

The idea that oxidative stress occurs with TNF-α treatment of cells is also supported by studies of oxidative stress-responsive transcription factors. It is increasingly evident that transcription factor activity may be modulated by the cellular redox state (39-43). Expression from transfected reporter constructs for the AP-1 and NF-κB transcription factors has been used as an *in vivo* indicator of oxidative stress (44,45). TNF-α treatment of the J. Jhan human lymphoblastoid T cell and U937 myelomonocytic cell lines leads to activation of an NF-κB-CAT reporter construct (45); NF-κB is **activated** under conditions of oxidative stress (43). Using this same approach, we have found that TNF-α treatment of the MCF-7 breast cancer cell line results in activation of NF-κB (Fig. 5). *We interpret this result as evidence of oxidative stress, concomitant with TNF-α-treatment, in this model for apoptosis of human breast cancer cells.*

Purpose of the Present Work: Studies of the Antioxidant Defense During TNF-α-induced Apoptosis of Breast Cancer Cells

PURPOSE: Based on the observation that TNF-α treatment of MCF-7 cells leads to activation of the oxidative stress-responsive transcription factor, NF-κB, we predict that the cells are experiencing oxidative stress. The purpose of the first Specific Aim of the project is to test whether the oxidative stress is mediated by a decline in components of the cellular antioxidant defense, and whether it leads to oxidative damage in the cell.

METHODS OF APPROACH: The plan is to: 1) treat cultures of the human MCF-7 and BT-20 cell lines with human recombinant TNF-α (Boehringer Mannheim) at a range of concentrations (0-500 U/ml) and collect samples after 1, 3 and 5 days; 2) extract RNA from one set of samples and conduct Northern blot hybridization analysis of catalase, MnSOD, Cu,Zn-SOD, DT-diaphorase, thioredoxin and GST gene expression; 3) assay a second set of samples for activity of antioxidant defense enzymes, and 4) assay a third set of samples for evidence of oxidative damage. We inititated the studies with MCF-7 cells and began by measuring apoptosis, based on an increase in DNA fragmentation as function of the dose and length of exposure to TNF-α.

BODY

MEASURING APOPTOSIS OF MCF-7 CELLS IN RESPONSE TO TNF-α

We used an ELISA to measure apoptosis of MCF-7 cells in response to varying doses of, and exposure times to, TNF-α. The ELISA is based on the detection of histone-associated DNA fragments in supernates of cell lysates; it is available as a kit from Boehringer Mannheim. For the assay, cells were plated in 60 mm tissue cultures dishes and left to attach for approximately 7 h. Human recombinat TNF-α (Boerhringer Mannheim) was then added to a final concentration of 50, 200, 500 or 1000 U/ml. Samples were also left untreated, to serve as controls. Treatment times of 3 and 5 days were tested. For the 3 day treatment, the cells were plated in 5 mls of culture media (RPMI supplemented with 10% calf bovine serum, penicillin and streptomycin) and the TNF-α was diluted and added in an additional 0.5 mls of media. For the 5 day experiment, the cells were plated into 3 mls media, TNF-α was diluted and added in 0.5 mls media and after 3 days an additional 2 mls of TNF-α-containing media was added. This was done because of a concern with going 5 days without a media change, but not wanting to replace the media and possibly lose apoptotic cells. For the assay, the culture

supernates were harvested and the cells were washed with 3 mls PBS, which was pooled with the supernates. The cells were collected by centrifugation, resuspended in 1 ml media and the viable cell counts were taken, based on trypan blue dye exclusion. The cell mixtures were transferred to eppendorf tubes and processed for the ELISA, following the manufacturer's instructions. Each treatment was set up in triplicate and the assays were repeated at least once. These studies showed a steady increase in TNF-α-induced DNA fragmentation as a function of dose and time (Fig. 6).

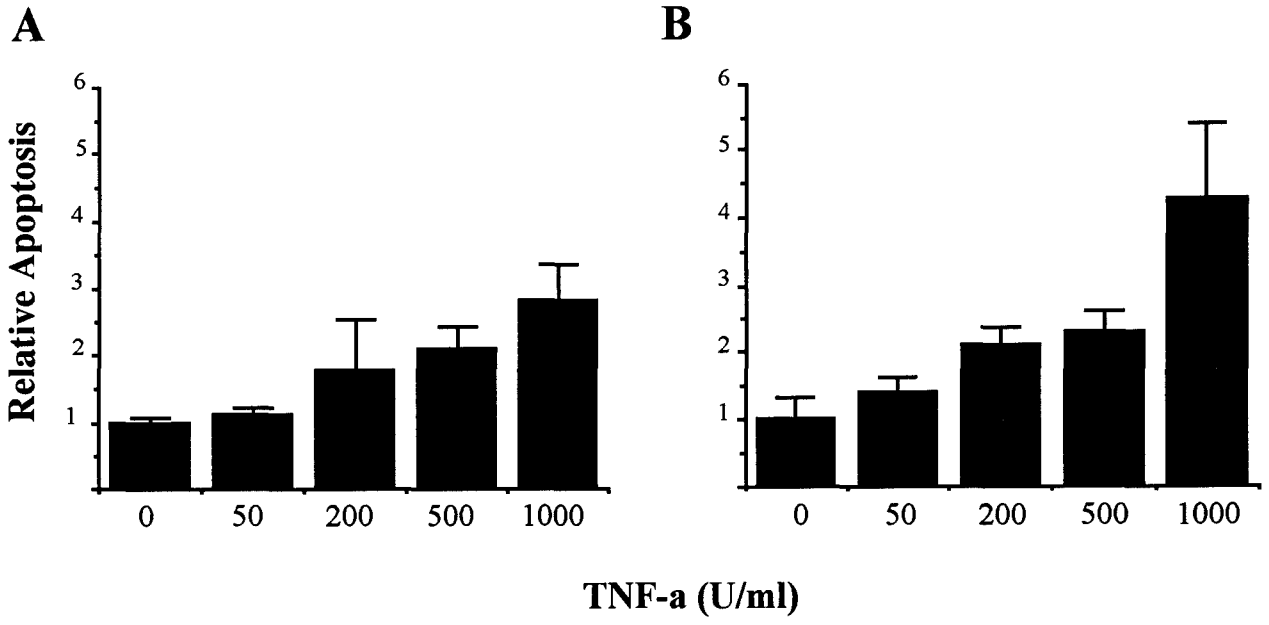


Figure 6. Apoptosis in MCF-7 cells in response to TNF-α. Cells were treated with the inidcated concentrations of TNF-α for 3 (A) or 5 (B) days. Apoptosis was measured using an ELISA for histone-associated DNA fragments. Values are relative to the untreated control and are the mean of two experiments, with each treatment done in triplicate (bars indicated S.E.M.).

NORTHERN BLOT HYBRIDIZATION ANALYSES

These experiments consisted of: 1) preparing Northern blots, 2) purifying cDNA sequences of MnSOD, CuZnSOD, DT-diaphorase, thioredoxin and glyceraldehyde 3-phosphate dehydrogenase (the latter is used as a standard), and 3) probing the blots with radiolabeled cDNA sequences.

Before setting up the Northorn blot hybridization analyses with RNA extracted from TNF-α-treated MCF-7 cells, I first trained the technician in the technique using RNA which was available from previous experiments (with WEHI7.2 cells). Northern blots were prepared by electrophoressis of RNA samples (15 μg/lane) through agarose gels containing formaldehyde and transferring the RNA onto nylon membranes by blotting. The RNA was UV crosslinked to the membranes. The cDNA sequences were purified from their respective cloning vectors by appropriate restriction endonuclease digestions, electrophoresis through low melting temperature agarose and purification of the cDNA by GeneCleaning (Bio101). Probe sequences were labeled by random primed synthesis.

purified from their respective cloning vectors by appropriate restriction endonuclease digestions, electrophoresis through low melting temperature agarose and purification of the cDNA by GeneCleaning (Bio101). Probe sequences were labeled by random primed synthesis.

Unexpectedly, for the first set of blots a signal was seen with only one of the probes. In troubleshooting experiments, it was discovered that the labeled cDNA sequences for this probe ran as a discrete band. The remaining probes had significant smearing (Fig. 7a). I then realized that this particular probe was synthesized using cDNA that I had purified in a former lab: GST cDNA described in the experiments above; a large amount of material was left from the previous experiments and I had decided to use it up, rather than make a new cDNA prep. I reasoned that the smearing seen on the autoradiograph might be indicating that the cDNA templates were damaged, possibly by the UV transilluminator used to visualize the DNA as it was excised from the gel. The UV transilluminator used to prepare the GST cDNA could be switched to 360 nm wavelength mode to visualize DNA for preparative workup, with minimal damaging effect. The UV transilluminator that is available to us in the Department of Pathology operates at 300 nm.

To test whether the UV transilluminator could be the source of the problem, a sample of the GST cDNA was exposed on the box for 2 minutes and then used as template in a labeling reaction. This treatment generated probe sequences that ran as a smear (7b). When this probe was tested against a Northern blot, it was found to hybridize much less efficiently than had been seen with the first GST probe. I conclude that the problem with the first set of Northern blots was largely due to taking too much time to excise the cDNA insert bands, while exposing the gel to UV light. We are in the process of repeating the process of purifying the cDNA sequences, in light of this finding. To minimize DNA exposure, a small amount of the restriction digestion is run in one lane, which is used to determine the position of the insert band on the gel. To protect the DNA in the remaining lanes, we place several layers of aluminum foil between the gel and the UV box.

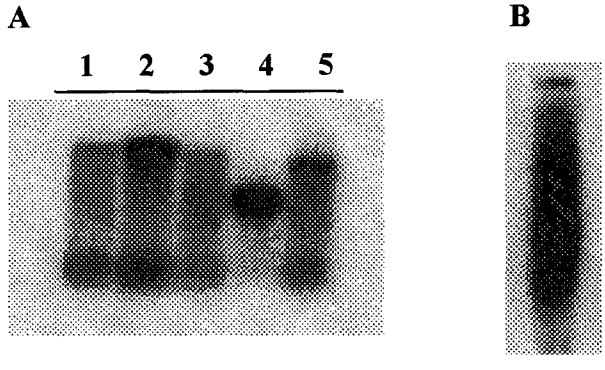


Figure 7. Overexposure to UV results in smearing of radiolabeled cDNA sequences. Autoradiograph of cDNA probes run out on an agarose gel A) Lane 1, catalase; lane 2, DT-diaphorase; lane 3, glyceraldehyde 3-phosphate dehydrogenase; lane 4, GST, and lane 5, MnSOD. The GST cDNA was exposed to a UV transillumator with 360 nm source; the remaining cDNAs were exposed to a 300 nm source. B) Autoradiograph of GST cDNA that was radiolabeled after exposure to the 300 nm UV light for 2 min.

For Northern blot analysis of TNF-α-treated MCF-7 cells, total RNA was extracted from cells after 3 days of treatment. The cells had been seeded into 150 mm dishes (2 dishes each for the treatment doses of 0, 50, 200 and 500 U/ml and 4 dishes for the 1000 U/ml treatment). A seeding density of 6.3 x 10^6 cells/dish was used. After attaching for approximately 7 h, TNF-α was added. After an additional 3 days, the culture media was collected and the cells were lysed by adding a guanidine isothiocyanate solution directly to the dish. The lysates were collected by scraping the plates with a rubber spatula, shearing the solution by passing it through a 20 gauge needle and then transferring it to the tubes in which the pellets from the media had been collected by centrifugation. Total RNA was then extracted from the samples by centrifugation through CsCl gradients. The following yields (based on absorbance at 260 nm) were obtained:

Treatment (U/ml)	0	50	200	500	1000
Yield (μg)	322	210	204	201	312

Since twice as many cells were treated at the 1000 U/ml dose of TNF-α, the lower per/cell yield for this treatment is consistent with cell loss by apoptosis. By running samples of the RNA (1 μg/lane) out on a 1% agarose gel (without formaldehyde, but run at ≈ 18 V/cm of gel), the technician confirmed that it was undegraded (Fig. 8).

Lane	Sample
1	Untreated
2	TNF-α 50 U/ml
3	TNF-α 200 U/ml
4	TNF-α 500 U/ml
5	TNF-α 1000 U/ml
MW	Kb ladder

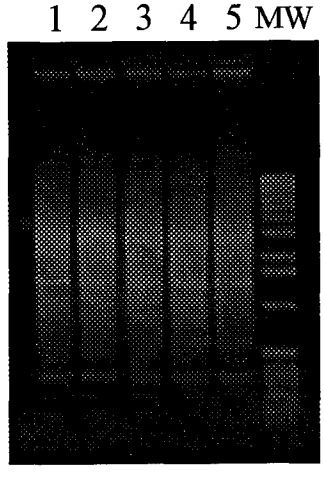


Figure 8. Agarose gel of total RNA extracted from MCF-7 cells after treatment with TNF-α for 3 days, at the indicated concentrations.

As the final step, prior to the Northern blot hybridization analyses of antioxidant defense enzyme expression in TNF-α-treated MCF-7 cells, the technician prepared mini-Northern blots and probed these with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA sequences. A new batch of purified GAPDH cDNA sequences had been prepared; the DNA was shielded from UV exposure during the workup. GAPDH sequences will be used against the final blots, to control for gel loading and transfer. Again, the goal of these experiments was to familiarize the technician with the techniques. These experiments demonstrated very consistent signals across the different treatment lanes (Fig. 9). I conclude that the problem with getting no signal on the first

set of blots has been resolved and anticipate being able to move very quickly to the completion of the proposed Northern blot hybridization analyses.

Lane	Sample
1	Untreated
2	TNF-α 50 U/ml
3	TNF-α 200 U/ml
4	TNF-α 500 U/ml
5	TNF-α 1000 U/ml

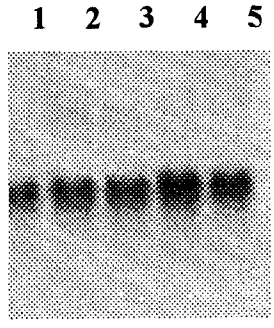


Figure 9. Northern blot hybridization analysis of GAPDH expression in TNF-α treated MCF-7 cells. Total RNA was extracted from cells after treatment for 3 days with the indicated concentrations of TNF-α and used to prepare a Northern blot (10μg RNA was loaded per lane). The blot was hybridized with ^{32}P-labeled GAPDH cDNA sequences.

BIOCHEMICAL ASSAYS FOR ANTIOXIDANT DEFENSES

For the studies of changes in the strength of the antioxidant defense,with TNF-α treatment of MCF-7 cells, we have begun by setting up the assay for catalase activity and testing various assays for reduced and oxidized GSH. The method of Lu *et al.* (46) was used to determine the specific activity of catalase standards (Sigma Chemical Co., St. Louis, MO) and the activity of catalase in the supernates of MCF-7 cells. The specific activity of the catalase standards was also measured by the method used by Sigma to determine activity units. Both methods are based on the decomposition of H_2O_2, which can be followed spectrophotometrically at OD_{240}.

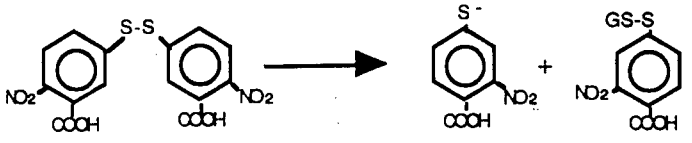
The kinetics of catalase do not follow the usual pattern of saturation of the enzyme by substrate. The decomposition of H_2O_2 by catalase is a first order reaction, the rate of which is always proportional to substrate concentration. Relatively low concentrations of catalase (0.06 - 0.03% v/v) must be used in the assay to avoid a rapid decrease in the initial rate of the reaction. The initial substrate concentration should be constant to assure reproducible results. This can be achieved by determining the absorbance of H_2O_2 stocks used in the assay. The decrease in absorbance at 240 nm should be measured within the first minute of the reaction. We found that only the initial rates of decomposition of H_2O_2 could be correlated to units of activity of the catalase standards. The effects of lysis buffer and freeze/thawing on the activity measurements were tested using the catalase standards. Lysis buffer appeared to lower the catalase activity to a small extent, but also prevented a small decrease in activity of the samples in phosphate buffer, that occurred upon freeze-thawing. We conclude that reliable measurements will be obtained when the cell samples, prepared in lysis buffer, are tested using catalase standards that are suspended in the same buffer. It will also not be a problem to assay the samples after storing them stored frozen.

Harvesting methods for the MCF-7 cells were also tested for the effect on the

disaggregated by trypsinization, washed three times with PBS and resuspended in the lysis buffer of Lu *et al.* (46). For the second harvesting method, the cell monolayers were washed three times with PBS and lysis buffer was added directly to the culture flasks. The cell lysates were collected by gentle scraping. Samples from both methods were incubated for 30 min. At 4°C and centrifuged at 3,000 x g for 15 min. The supernatant fractions were stored at -20°C before assaying for catalase activity. Identical values (6.42 μmoles/mg/min) were obtained for the two methods.

Most enzymatic methods for determination of total glutathione and oxidized glutathione are based on converting the oxidized form of glutathione (GSSG) to the reduced form (GSH) using glutathione reductase linked to the NADPH/NADP system. The stoichiometric decrease in NADPH that occurs during the conversion of oxidized to reduced glutathione (Reaction 1) can be monitored spectrophotometrically at 340 nm and therefore can be used specifically to measure levels of the oxidized form. Incorporation of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) into the assay system allows for the detection of both GSSG and GSH, since DTNB reacts with reduced glutathione and produces a chromophore that absorbs at 412 nm (Reaction 2).When GSSG is measured in the presence of GSH, the samples are first reacted with N-ethylmaleimide, a compound that blocks free sulfhydryls and prevents the oxidation of GSH during the sample workup or assay.

(1) GSSG + NADPH ⟶ 2GSH + NADP+

(2) GSH + 

We examined several versions of the assay described above. Results obtained using the method of Tietze (47) were not very reproducible and the blank was consistently high. The method of Akerboom and Sies (48) for total glutathione produced more consistent results with a substantially lower blank. Their method for GSSG appeared to work quite well. The GSH concentration in the assay mixture was measured upon complete conversion of a GSSG standard to GSH (Reaction 1). The assay mixture was incubated for 10 min at 65°C to denature glutathione reductase, cooled on ice and the GSH content was measured using the method of Ellman (49). There was good correlation between the decrease in absorbance at 340 nm and GSH concentration at the end of the reaction. However, a problem was observed with the total glutathione assay. Different reaction rates were observed, depending upon whether GSH or GSSG was used as a standard; the rate was higher when GSSG was used as the standard (Fig. 10). This difference makes it difficult to accurately determine GSH and GSSG levels, in mixtures of these two. A method for measuring GSH using methylglyoxal/glyoxylase 1 is being examined, as well as various HPLC techniques for GSSG, GSH and total glutathione.

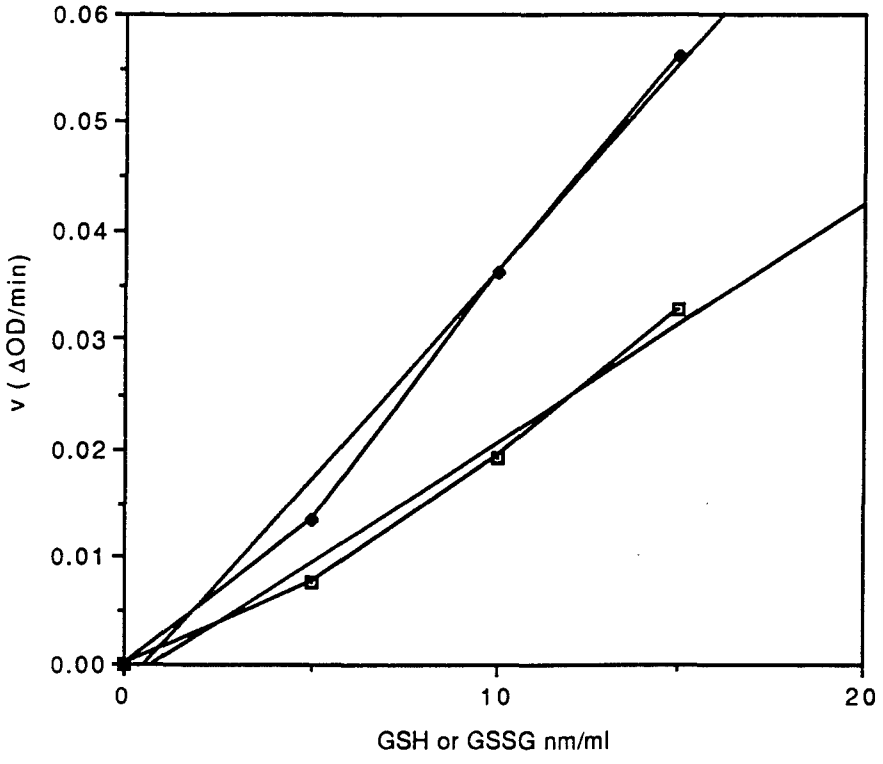


Figure 10. Dependence of the rate of reduction of DTNB, measured by the change in absorbance at 412 nm, on GSH or GSSG concentration. The method used to determine total glutathione in each sample was that of Akerboom and Sies (48). Reaction velocity (v) was measured at 5, 10 and 15 nmoles/ml of either GSH (❑) or GSSG (■). In each case, the straight line represents simple linear regression analysis.

CONCLUSIONS

Our studies during the past year have demonstrated dose- and time-dependent increases in apoptosis, as based on an ELISA for DNA fragmentation, with TNF-α treatment of MCF-7 cells. In setting up Northern blot hybridization analyses, we encountered problems that appear to be due to UV damage to the cDNA sequences, that were used to prepare the radiolabeled probe sequences. By devising a way to shield the sample from UV exposure during the workup procedure, we resolved the problem. The technician has now been trained in the Northern blotting technique, as indicated by the quality of her recent blots of RNA from a 3 day TNF-α treatment series. For the measurements of the antioxidant defense, following TNF-α treatment of MCF-7 cells, we have a catalase assay underway and are in the process of comparing methods for measuring GSH content.

Overall, the project is not as far along as anticipated. This was first of all due to my carrying through on commitments made prior to the start of the Army project. Significant effort was put into an invited review paper entitled, "Redox Signalling and the Control of Cell Growth and Death", for the journal *Pharmacology and Therapeutics* (a copy is included in the Appendix). I also contributed to additional

Therapeutics (a copy is included in the Appendix). I also contributed to additional studies of oxidative stress in dexamethosone-induced lymphocyte apoptosis that have been submitted for publication (see Appendix: Baker *et al.*, "Decreased Antioxidant Defense and Increased Oxidant Stress During Dexamethasone-Induced Apoptosis: *bcl-2* Selectively Prevents the Loss of Catalase Activity"). At the request of my Department Head, I audited the general Pathology section (approximately 8 weeks) of the Pathologycourse for second year medical students. This given me a better understanding of cancer as a clinical entity. Due to my decision to meet these commitments (and process of writing a review paper taking more effort than I imagined), I delayed the search for a technician until this past Spring.

During the past year, I was encouraged to organize a campus-wide journal club focusing on apoptosis. The group has met once a month since last October and has grown to 50+ members, from basic science and clinical departments (the Directory of Members is included in the Appendix). The time that I spend organizing the presentations and ensuring that the articles are distributed to the participants is compensated by my increased opportunity to interact with other researchers conducting apoptosis research. I was also asked to organize basic scientists to present their research, relevant to breast cancer, at a day long workshop on the topic of breast cancer (a copy of the Workshop schedule is included in the Appendix). The conference was held Saturday, September 9, 1995 and was attended by clinicians, basic scientists, nurses and social workers with an interest or research experience in breast cancer.

In conclusion, my disappointment with not yet realizing the research goals set for the first year is tempered by my satisfaction with my continued development as a scientist in the breast cancer field. My goals for the coming year are to quickly bring the project up to schedule and to avoid additional commitments that will take time away from my research.

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APPENDIX

The Role Of Oxidative Stress in Apoptosis of Breast Cancer Cells

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REDOX SIGNALLING AND THE CONTROL OF CELL GROWTH AND DEATH

ED: charge sequence to *, †, ‡, §

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Abstract—Cells maintain a reduced intracellular state in the face of a highly oxidizing extracellular environment. Redox signalling pathways provide a link between external stimuli, through the flavoenzyme-mediated NADPH-dependent reduction of intracellular peptide thiols, such as glutathione, thioredoxin, glutaredoxin, and redox factor-1, to the posttranslational redox modification of certain intracellular proteins. This can affect the proteins' correct folding, assembly into multimeric complexes, enzymatic activity, and their binding as transcription factors to specific DNA sequences. Such changes have been linked to altered cell growth and death.

Keywords—Redox signalling, cell growth, thioredoxin, glutaredoxin, glutathione, transcription factors.

CONTENTS

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Abbreviations—AP-1, activator protein-1; BCNU, bis(2-chlorethyl)-nitrosourea; BSO, L-buthionine-(*SR*)-sulfoximine; DTT, 1,4-dithiothreitol; EGR-1, early growth response-1; EPF, early pregnancy factor; FSH, follitropin, follicle-stimulating hormone; Grx, glutaredoxin, thioltransferase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; HIV, human immunodeficiency virus; IκB, inhibitor of NF-κB; IL, interleukin; MnSOD, manganese superoxide dismutase; NAC, N-acetylcysteine; NEM, N-ethylmeleimide; NF-κB nuclear factor-κB; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; PDI, protein disulfide isomerase; PDTC, pyrrolidine dithiocarbamate; PKC, protein kinase C; REF-1, redox factor-1; ROS, reactive oxygen species; RR, ribonucleotide reductase; TNF-α, tumor necrosis factor-α; TPA, 12-O-tetradecanoylphorbol-13-acetate; TR, thioredoxin reductase; Trx, thioredoxin.

1. INTRODUCTION

The extracellular environment is predominantly oxidizing. An oxidized state within the cell would be extremely harmful due to reactions such as radical generation leading to lipid peroxidation, DNA crosslinking, and formation of disulfide bonds in proteins. Thus, cells have developed sophisticated mechanisms for maintaining an intracellular reduced state in the face of an oxidizing external environment. The intracellular reduced environment previously has been considered relatively constant unless a cell was undergoing an extreme stress response or damage (e.g., due to exposure to toxins, peroxides, and radiation). It is becoming clear that redox regulation is an important function in such biological events as DNA synthesis, enzyme activation, selective gene expression, and cell cycle regulation. That the cellular redox state can fluctuate between a more oxidized and reduced state suggests that this modulation can be regulated.

In this review, the level and components of the cellular machinery used for the redox regulation of protein activity are discussed. Until recently, this form of posttranslational modification has received much less attention than protein phosphorylation. Redox modification of proteins is confined to the amino acid cysteine, whereas phosphorylation can occur on either serine, threonine, tyrosine, or histidine residues. Phosphorylation is more likely to have an effect by acting as a prosthetic group for protein recognition by another protein. Redox events, on the other hand, are more likely to impart a structural change to the target protein, rendering the protein more or less active. As with any biological system, variations from this theme can occur.

This review will focus on the known major redox signalling systems that regulate proliferation and death in eukaryotic cells, namely, glutathione/glutathione reductase, thioredoxin (Trx)/thioredoxin reductase (TR), glutaredoxin (thioltransferase, Grx), and the recently discovered Ref-1 protein. Interrelations among these redox systems and some of their functions in the cell are shown in Fig. 1.

2. EUKARYOTIC CELLULAR REDOX SYSTEMS

2.1. Glutathione

2.1.1. Overview

The reduced glutathione (GSH)/oxidized glutathione (GSSG) couple is the major redox buffer in the cell (for reviews, see Meister and Anderson, 1981; Kosower and Kosower, 1978). GSH is a tripeptide of γ-glutamate, cysteine, and glycine, which is found ubiquitously in eukaryotic cells at a concentration between 1 and 10 mM (Kosower and Kosower, 1978). The intracellular environment is highly reducing due to the GSH/GSSG couple lying predominantly in favor of GSH; under physiological conditions, ratios of 30:1-100:1 have been measured (Hwang *et al.*, 1992). A reducing environment in this range presumably is needed to counteract oxidants produced during normal metabolism and for reductive biosynthesis.

Cellular GSH content is determined by the rates of import and synthesis vs. the rates of efflux and conjugation. Most cell types don't import GSH (Meister, 1991), and synthesis occurs intracellularly

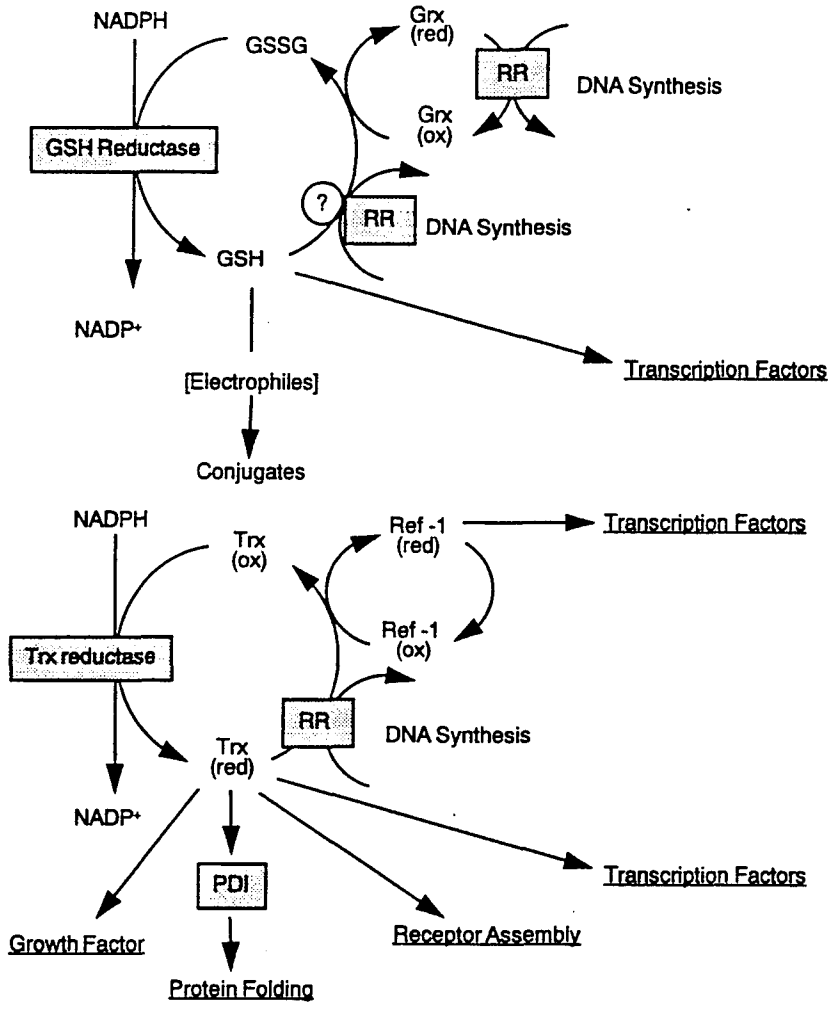

Fig. 1. Redox systems and their effects on processes regulating cell growth. Reducing equivalents from NADPH are transferred to GSSG or to oxidized Trx [Trx(ox)], by glutathione reductase and TR, respectively, giving GSH and reduced Trx [Trx(red)]. GSH directly reduces oxidized Grx [Grx(ox)], and Trx reduces oxidized Ref-1 [Ref-1(ox)]. Trx and Grx provide reducing equivalents to RR for deoxyribonucleotide synthesis. A third Grx-like, or other protein thiol, may transfer reducing equivalents to RR, here shown from GSH. GSH protects the cell against reactive electrophiles by forming conjugates. Trx reduces critical protein sulfhydryl residues to regulate protein folding, receptor assembly, and transcription factor activity, and also acts as a growth factor. Protein folding is catalyzed by PDI, which has Trx-like sequences. Grx(red) is reduced Grx; Ref-1(red) reduced Ref-1.

in sequential, ATP-dependent reactions catalyzed by γ-glutamylcysteine synthetase and GSH synthetase. The rate of synthesis is influenced primarily by the availability of cysteine, but also by feedback inhibition of synthesis by GSH. Conversion of GSH to its oxidized form, GSSG, occurs during GSH peroxidase-catalyzed reduction of hydrogen peroxide (H_2O_2) and other peroxides, and in spontaneous reactions with free radicals (Meister, 1994). GSSG is restored to a reduced form by the flavoprotein glutathione reductase (Lopez-Barea *et al.*, 1990). Irreversible loss of GSH occurs through conjugation to endogenous and exogenous electrophilic centers in reactions catalyzed by GSH transferases (Pickett and Lu, 1989; Morrow and Cowan, 1990). The conjugates subsequently are excreted as mercapturic acids. GSH turnover is also found in association with the import of γ-glutamyl amino acids and with the intercellular transport of cysteine (Meister, 1994).

A number of methods have been used experimentally to modulate intracellular GSH content. Ideal use of this approach for examining the role of the GSH/GSSG redox couple in protein regulation considers the possibility that GSH, GSSG, or both may be important. Methods used to increase

GSH content include enhancing synthesis by the addition of precursors, including the cysteine analog L-2-oxothiazolidine-4-carboxylate (Williamson *et al.*, 1982) or GSH ester (Levy *et al.*, 1993). For cells able to import GSH, intracellular GSH content may be enhanced by addition of GSH to the media (Lash *et al.*, 1986). GSH content can be lowered by irreversibly inhibiting γ-glutamylcysteine synthetase with L-buthionine-(*SR*)-sulfoximine (BSO) (Meister, 1988), increasing conjugation and efflux by diamide treatment (Reed, 1986), or by limiting the supply of cysteine. Levels of intracellular GSSG may be increased through inhibition of glutathione reductase with bis(2-chlorethyl)-nitrosourea (BCNU) (Babson *et al.*, 1981).

The reducing activity of GSH is provided by the thiol group present on the cysteine moiety. Redox reactions in which the GSH/GSSG redox couple play a role include protein folding (Hwang *et al.*, 1992), conversion of ribonucleotides to deoxyribonucleotides (Holmgren, 1990) and maintenance of reduced pools of vitamins C and E (Winkler *et al.*, 1994). GSH can also undergo reversible thiol-disulfide exchange with proteins containing oxidized cysteine (cystine) groups (Ziegler, 1985). As discussed in the next section, GSH may also play a direct role in cellular signalling through thiol-disulfide exchange reactions with membrane-bound receptor proteins, transcription factors, and regulatory proteins in the cell.

2.1.2. *Effects on Receptor Proteins*

Thiol-disulfide exchange reactions involving GSH may be important for insulin-induced cell signalling by stabilizing and activating the insulin-receptor complex (Cotgreave *et al.*, 1994). Site-directed mutagenesis of the lymphokine interleukin (IL)-2 has demonstrated the importance for biological activity of a cysteine-containing region in the protein (Liang *et al.*, 1986). Pretreatment of the IL-2-dependent CTLL-2 and CT-4R cell lines with GSH enhances IL-2-receptor binding and internalization and DNA synthesis (Liang *et al.*, 1989). Pretreatment of the cells with the GSH synthesis inhibitor BSO attenuates the increase in DNA synthesis in response to IL-2.

2.1.3. *Effects on Transcription Factors*

Studies by Galter *et al.* (1994) suggest that regulation of gene expression by redox-sensitive transcription factors depends on the balance of pro-oxidant and antioxidant conditions, particularly of GSH and GSSG, in the cell. Both the nuclear factor-κB (NF-κB) and Activator protein-1 (AP-1) transcription factors were shown to be activated upon treatment of cells with BCNU, a compound that, among other actions, elevates intracellular levels of GSSG by inhibiting glutathione reductase (Galter *et al.*, 1994). The DNA binding activity of the factors was shown, by gel shift assays and transfection studies with reporter constructs, to be inhibited by GSSG and oxidized Trx (see Section 2.2.1). NF-κB DNA binding activity was restored by incubation of the nuclear extracts with reduced Trx. The authors concluded that optimal induction of NF-κB functions may even require a shift from high reduced GSH/low Trx conditions to low reduced GSH/high Trx levels (Galter *et al.*, 1994). Examination of the compartmentalization of these compounds between the cytoplasm and nucleus, under physiological conditions of NF-κB activation, is needed to test this possibility. A reduction in DNA binding in the presence of GSH has also been reported for the transcription factor Sp1 (Knoepfel *et al.*, 1994). The effect on DNA binding was much less pronounced when DNA binding was carried out prior to the incubation with GSH, suggesting that the sensitive thiol groups are protected by DNA binding.

2.1.4. *Effects on Gene Expression*

Heme oxygenase is induced, through a change in gene transcription, following exposure of cells to H_2O_2 or ultraviolet A radiation (Keyse and Tyrell, 1987). Inhibition of GSH synthesis by BSO treatment of the human skin fibroblast cell line FEK4 leads to a higher basal level and enhanced expression of heme oxygenase in untreated and ultraviolet A-irradiated cells, respectively (Lautier *et al.*, 1992). Interestingly, a drop in the level of induction was observed at the highest level of GSH depletion, reminiscent of a similar effect of GSH on NF-κB activation.

2.1.5. *Other Effects of Glutathione Depletion*

Studies of changes in cell metabolism, subsequent to oxidant damage, have established a role for GSH in Ca^{2+} signalling (Jewell *et al.*, 1982; Bellomo *et al.*, 1982; Jones *et al.*, 1983). Incubation of isolated hepatocytes with *t*-butyl hydroperoxide leads to depletion of GSH and NADPH, release of Ca^{2+} from mitochondrial and extramitochondrial stores, and blebbing of the cell surface (Jewell *et al.*, 1982; Bellomo *et al.*, 1982). Preincubation of the cells with the thiol compound 1,4-dithiothreitol (DTT) prevents these effects, while inhibition of glutathione reductase accelerates the response. However, in the latter experiments, Ca^{2+} was released only from the extramitochondrial stores. Thus, it appears that the mechanism of Ca^{2+} efflux from these sites, chiefly the endoplasmic reticulum, is particularly sensitive to changes in cellular redox state (Jones *et al.*, 1983). From studies of the release of Zn^{2+} from metallothionine in response to incubation with GSSG, it has been suggested that GSH redox status may influence cellular processes requiring Zn^{2+} (Maret, 1994).

In lymphocytes, the proliferative response following mitogenic stimulation is highest in cells having a high intracellular GSH content (Kavanagh *et al.*, 1990). On the other hand, terminal differentiation may also require a minimum GSH content, since phorbol ester-induced differentiation of human myeloid cell lines is blocked when the cells are depleted of GSH (Esposito *et al.*, 1994). In this case, DNA binding of the AP-1 and early growth response-1 (EGR-1) transcription factors, in response to phorbol ester, does not occur in the GSH-deficient cells, suggesting that this step in the growth-signalling pathway is regulated by the redox state of the cell.

Active GSH redox cycling may inhibit cell growth indirectly by drawing on the pool of reducing equivalents in the cell. Most cellular products are more reduced than their substrates, and biosynthesis is, thus, in general, a reductive process. Reducing equivalents for the conversion of GSSG to GSH are provided by NADPH. Thus, both reductive biosynthetic reactions and the glutathione redox cycle draw on the cellular NADPH pool. Since glutathione reductase consumes NADPH at a higher rate than other NADPH-dependent enzymes (Reed, 1986) and there is approximately 10 times more GSH than NADPH in cells, active GSH redox cycling can be expected to occur at the cost of limiting biosynthetic reactions in the cell.

2.2. Thioredoxin Reductase/Thioredoxin

The TR/Trx couple constitutes a ubiquitous redox system found in prokaryotic and eukaryotic cells. TR is a flavin adenine dinucleotide-containing flavoenzyme that uses NADPH as a source of reducing equivalents. Cycling of oxidized TR to the reduced form requires electron transfer from NADPH. The flavin adenine dinucleotide prosthetic group, in turn, transfers the electrons to active site cysteines, resulting in a catalytically active form of TR, capable of reducing the active site cystine bond of oxidized Trx.

Bacterial TR was purified from *Escherichia coli* almost 30 years ago and found to be a homodimer comprised of two 35 kDa subunits (Thelander, 1967). TrxB, the gene encoding for *E. coli* TR, has been cloned (Russel and Model, 1985), sequenced (Russel and Model, 1988), and its physical location identified (Delaney and Georgopoulos, 1992). Several studies have examined the biochemical properties of the active site and substrate specificity of this flavoenzyme. Cys-135 and Cys-138 were identified as the active-site cysteine residues that receive electrons from reduced flavin adenine dinucleotide and transfer them to Trx (Prongay *et al.*, 1989). Subsequent reports identified Cys-136 as interacting with the flavin moiety (Prongay and Williams, 1990) and calculated the redox potentials of the active site of bacterial TR (Delaney and Georgopoulos, 1992). A site-directed mutagenesis study of His-245 and Asp-139 revealed Asp-139 as the active-site acid catalyst for reduction of Trx (Mulrooney and Williams, 1994). Stopped-flow kinetics analysis have shown that the formation of the Trx-TR complex occurs rapidly (Navarro *et al.*, 1991). Subsequent electron transfer to Trx's active site occurs rapidly and the entire reaction is independent of substrate (Trx) concentration.

It was not until 1989 that the tertiary structure of *E. coli* Trx was elucidated by X-ray crystallography (Kuriyan *et al.*, 1989) and a refined structure developed (Kuriyan *et al.*, 1991). An observation was that the sequence and structure of bacterial TR had a high degree of similarity with human glutathione reductase. Recent work has suggested a mechanism of action for TR involving key structural rearrangements. During reduction, TR undergoes a conformational change that protects the reduced active-site cysteines from the aqueous phase, preventing spontaneous oxidation. Upon binding of

oxidized Trx to the active site, TR alters its structure to expose the active-site cysteines, allowing reduction of Trx's cysteine bond (Waksman *et al.*, 1994). In addition, this work has made more clear the differences between bacterial TR and human glutathione reductase.

Eukaryotic TR has been purified as a 116–120 kDa homodimer from numerous mammalian sources, including calf thymus (Luthman and Holmgren, 1982), human placenta (Oblong *et al.*, 1993), and porcine organs (Kistner *et al.*, 1993). Recent work has found that penicillin synthesis in *Penicillium chrysogenum* requires the Trx/TR system (Cohen *et al.*, 1994). Purification of the proteins of this redox system and cloning revealed a 26–60% sequence identity with bacterial Trxs and TR. This is the first example of the cloning of a eukaryotic TR gene. Studies of human TR have shown that it can reduce bacterial Trx, although at a decreased rate compared with human Trx (Oblong *et al.*, 1993). The converse has been found for bacterial TR using human Trx, where the bacterial homolog was a better substrate than mammalian Trx (Jacquot *et al.*, 1990).

There are a number of reported inhibitors of TR that were originally studied because of the possibility that the Trx/TR redox system could ~~regulate~~ DNA synthesis through its regulation of ribonucleotide reductase (RR) (for a discussion of Trx and RR, see Section 2.2.2). Inhibitors of TR include anthralin (Schallreuter and Pittelkow, 1987), azelaic acid, which is a competitive inhibitor (Schallreuter and Wood, 1987), and mechanism-based (suicide) substrate inhibitors, which include the antitumor quinones doxorubicin and diaziquone (Mau and Powis, 1992a), antitumor nitrosoureas (Schallreuter *et al.*, 1990), and 13-*cis*-retinoic acid (Schallreuter and Wood, 1989). In contrast, alkyl 2-imidazolyl disulfide analogues are inhibitors of TR that do not show the characteristic time dependence of mechanism-based inhibition (Oblong *et al.*, 1994c). With the recent evidence that the Trx/TR redox system may function as a growth regulator through systems in addition to RR, there is continued interest in inhibiting TR as a means of blocking unregulated cell growth.

2.2.1. *Thioredoxin*

Trx is an 11–12 kDa protein with an active site that is highly conserved among bacterial, plant, and vertebrate forms, having a consensus sequence Trp-Cys-Gly/Pro-Cys-Lys. The two half-cysteines at the active site of oxidized Trx are reduced in an NADPH-dependent reaction catalyzed by TR, the final product of which, reduced Trx, can reduce protein disulfides. Mammalian Trxs have been cloned from numerous sources, either directly or through a genetic screening methodology. A single copy of the human Trx gene has been mapped to chromosome 3 at bands 3p11–p12 (Lafage-Pochitaloff-Huvale *et al.*, 1987). Analysis of genomic clones of Trx have found that the entire gene spans 13 kb and is comprised of 5 exons (Tonissen and Wells, 1991; Kaghad *et al.*, 1994). In contrast, a murine Trx pseudogene has been identified on chromosome 1 in addition to a single copy of Trx mapping to chromosome 4 (Taketo *et al.*, 1994). While only one Trx gene has been identified in human and murine genomes, it has been suggested on the basis of isoelectric focussing that there are two separate forms of bovine Trx (Martinez-Galisteo *et al.*, 1993). A slightly larger mitochondrial form of Trx, compared with cytosolic Trx, has been identified in pig heart based on electrophoretic mobility (Bodenstein-Lang *et al.*, 1989; Bodenstein and Follmann, 1991). However, small differences in electrophoretic mobility could be due to posttranslation modifications, such as phosphorylation of Trx, which has been detected in *E. coli* (Conley and Pigiet, 1978). In yeast, two Trx genes, TRX1 and TRX2, have been mapped to chromosome XII and VII, respectively (Muller, 1992).

The amino acid sequences of the active sites are highly conserved between bacterial and human Trxs. A number of mutagenesis studies have been reported characterizing Trx's conserved active-site sequence. A residue that has drawn particular attention is a highly conserved lysine group adjacent to the *C*-terminal-most active-site cysteine. The presence of the positively charged amine group so close to the active site has lead to the suggestion that it is critical in maintaining the thiolate anion of Cys-32 (Kallis and Holmgren, 1980). However, pH titration curves of human Trx analyzed by NMR spectroscopy have shown that the thiolate form of Cys-32 is stabilized by interaction of the γ-S of Cys-32 with the peptide backbone amide group of Cys-35 (Forman-Kay *et al.*, 1992). Furthermore, mutagenesis of Lys-36 to Glu in both *E. coli* (Gleason *et al.*, 1990) and human Trx (Oblong *et al.*, 1995) did not affect the ability of the mutant to be redox active. Thus, the highly conserved lysine group adjacent to the active site is not essential for the reduction of either prokaryotic or eukaryotic Trx by TR, but is seemingly required for optimizing protein interactions with the

flavoenzyme, as evidenced by the increase in the K_m values for the mutant Lys-36 human Trx (Oblong *et al.*, 1995).

Trx is an unusually thermostable protein. *E. coli* Trx is very resistant to thermal denaturation (Bala *et al.*, 1990), with TM values of 86°C and 75°C for the oxidized and reduced species, respectively (Russel and Holmgren, 1988). Trx isolated from *Sulfolobus solfataricus*, a thermophilic bacteria, was found to have extreme heat stability, withstanding temperatures of 90°C for hours without apparent loss of activity (Guagliardi *et al.*, 1994). Thermal stability may be a shared property with eukaryotic Trxs, since recombinant human Trx can be incubated at 80°C for several hours with no apparent decrease in catalytic efficiency in the insulin reduction assay (Oblong *et al.*, 1993).

2.2.2. *Cofactor for Ribonucleotide Reductase*

One of the earliest cellular functions ascribed to bacterial Trx was the redox regulation of RR. RR catalyzes the synthesis of deoxyribonucleotides from ribonucleotides, which is the first unique reaction of DNA synthesis and an essential step for cellular proliferation (Laurent *et al.*, 1964). Thus, Trx has been presumed to function as a key component in DNA synthesis through its ability to reduce RR. It was presumed that after each reaction, RR had to be reduced by Trx to become active. This, in turn, led to the hypothesis that Trx was essential for cell viability. However, studies of a mutant of *E. coli* lacking Trx that was able to proliferate led to the identification of Grx, another redox active protein with similar properties to Trx (Holmgren, 1976). Double *E. coli* mutants lacking both Trx and Grx will not proliferate due to the accumulation of adenosine 3′-phosphate 5′-phosphosulfate (PAPS), an intermediate in the sulfate assimilation pathway (Russel and Holmgren, 1988, 1990). Inhibiting the formation of PAPS by the use of high concentrations of cystine, or mutations in the genes required for PAPS synthesis, overcomes the inhibition of growth (Russel and Holmgren, 1990). This indicates that there may be a third redox component in bacteria involved in the essential redox regulation of RR and DNA synthesis. Recent studies have suggested that this occurs in a GSH-dependent manner (Miranda-Vizuete *et al.*, 1994), and two proteins with Grx-like activity and typical active sites of Cys-Pro-Tyr-Cys, of 10 and 27 kDa, have been isolated from the *E. coli* mutant (Aslund *et al.*, 1994).

In yeast, it has been shown that Trx is not essential for RR activity since yeast mutants lacking both copies of the Trx gene were found to be viable (Muller, 1991). However, the absence of Trx led to an increase in the mitotic cycle and DNA replication rates.

The importance of Trx for eukaryotic RR is less well understood. Since there is a lack of genetic and molecular evidence, as exists for bacterial Trx, it is difficult to speculate on whether or not Trx is essential for DNA synthesis. Indirect evidence suggests that inhibition of TR by antitumor quinones leads to a decrease in the activity of RR (Mau and Powis, 1992b). A recent report has identified a homolog of Trx in *Drosophila* (Salz *et al.*, 1994). The homolog is encoded in the *deadhead* locus. Mutation of the locus revealed that the Trx homolog was not essential for cell viability, but was essential for female meiosis.

2.2.3 *Cofactor for Vitamin K Metabolism*

Vitamin K-2,3-epoxide reductase has been shown to require reduced Trx as a cofactor for activation (Silverman and Nandi, 1988). Trx was considerably more potent than DTT in activating the reductase, suggesting a possible physiological role for Trx as a cofactor for the enzyme.

2.2.4. *Effect on Receptor Proteins*

The ability of Trx to reduce and activate receptors such as the glucocorticoid receptor (Grippo *et al.*, 1983) and the interferon-γ receptor (Fountoulakis, 1992) has led to the identification of Trx-like active-site domains in gonadotropic hormones. The β-subunits of both follitropin (FSH) and luteotropin were found to not only contain a Trx active-site domain, but to be capable of reactivating reduced and denatured ribonuclease (Boniface and Reichert, 1990). In subsequent work, 20-amino-acid peptides of this domain were shown to bind to human FSH receptors and to propagate a signalling effect characteristic of human FSH. Substitution of the active site cysteines with serines did not block the ability of the peptides to bind to the receptors, but did abolish the transduction of the signal

across the membranes (Grasso *et al.*, 1993). It is not known if these hormones interact with their respective receptors and initiate a ligand-receptor response through a mechanism that involves a redox reaction similar to that catalyzed by Trx.

2.2.5. *Protein Folding and Degradation*

Trx is capable of catalyzing the *in vitro* folding of proteins by a mechanism similar to that of protein disulfide isomerase (PDI) (Lundström and Holmgren, 1990), which contains two Trx-like active-site domains (for a review, see Noiva and Lennarz, 1992). As has been shown with rat prolactin, reduction by Trx can so alter protein structure as to render the protein susceptible to proteolysis. Rat glandular kallikrein (a serine-type protease) was capable of cleaving Trx-treated prolactin *in vitro* to a form found to be present *in vivo* (Hatala *et al.*, 1991). Trx has been found to be active in reducing toxic venoms from such species as scorpion, wasp, and snakes (Lozano *et al.*, 1994). This activity may lead to increased susceptibility of the toxins to proteolysis. Tetanus toxin was found to have diminished toxicity when the interchain disulfide bond was reduced by Trx (Kistner *et al.*, 1993). Oxidized Trx reformed the cleaved interchain disulfide bond, thus restoring full potency to the tetanus toxin. A point to be stressed in this work is that Trx was specific as a reducing agent and other dithiol reagents, such as GSH or DTT, were without effect.

2.2.6. *Thioredoxin in the Early Pregnancy Factor Complex*

Trx has been identified as a component in the early pregnancy factor (EPF) system, a complex array of factors present in the sera of pregnant mammals (Clarke *et al.*, 1991). The binding of lymphocytes to red blood cells, i.e., rosette bud formation, by EPF occurs during the initial onset of pregnancy, and several proteins of the EPF complex may act synergistically or in combination. Trx was identified as a component of EPF, which contributed to, but was not essential for, the biological response (Clarke *et al.*, 1991). A mutagenesis study of human Trx showed that the redox active, catalytic site Cys-32 and Cys-35 residues were not essential for this function, but that Cys-73 was (Tonissen *et al.*, 1993). The latter is one of three nonactive site cysteine residues (the others are Cys-62 and Cys-69), all of which have been suggested to function in a structural capacity in eukaryotic homologs (Holmgren, 1985). A recent report has refuted the hypothesis of Trx being EPF, suggesting instead that chaperonin 10 is EPF (Cavanaugh and Morton, 1994). A possible explanation for this discrepancy is that both Trx and chaperonin 10 are present in EPF.

2.2.7. *Other Effects*

Trx was originally identified as an IL-2 receptor-inducing factor in lymphoid cells (Yodoi *et al.*, 1984; Tagaya *et al.*, 1988; Wollman *et al.*, 1988). Gene transcription of the IL-2 receptor is increased by Trx in human T-cell lymphotropic virus Type 1 transformed T-cells (Tagaya *et al.*, 1989; Teshigawara *et al.*, 1985; Yodoi *et al.*, 1984). Addition of Trx to the medium protects embryo cells *in vitro* during their early development (Natsuyama *et al.*, 1993). Trx will also protect cells against the toxicity of some venoms (Lozano *et al.*, 1994) and against tumor necrosis factor-α (TNF-α) cytotoxicity (Matsuda *et al.*, 1991). The choline acetyltransferase activity of neuronal cells is increased by Trx (Lowe *et al.*, 1993).

2.3. Protein Disulfide Isomerase

PDI is an approximately 57 kDa protein localized to the endoplasmic reticulum, where it comprises 0.4% of the total protein. It has been suggested that PDI's principal function is to catalyze the proper folding of proteins during maturation and secretion from the endoplasmic reticulum (Edman *et al.*, 1985). The extremely reducing environment of the endoplasmic reticulum, regulated by the GSH/GSSG ratio, has an impact on the active state of PDI. The *E. coli* equivalent, dsbA, is considerably smaller in size than PDI (approximately 23 kDa) and performs disulfide bond formation in the periplasmic space of the bacterium (Wunderlich and Glockshuber, 1993). In the lower eukaryote yeast, PDI is present as a 70 kDa protein (Mizunaga *et al.*, 1990) and is essential for cell viability (Farquhar *et al.*, 1991).

PDI contains two Trx-like active sites, both of which are biochemically functional. The active site sequence of PDI, Cys-Gly-His-Cys-Lys, varies from Trx only by substitution of His for Pro, which is present in both the bacterial and human Trx. An important finding was that the Trx-like active sites in PDI are capable of being reduced by TR, as evidenced by PDI being able to substitute for Trx in the Trx-selective insulin reduction assay (Lundström and Holmgren, 1990). Mutagenesis of the active site of Trx to the PDI sequence generated a mutant Trx that was actually a better substrate for TR (Krause *et al.*, 1991) and had a 10-fold increase in its PDI-like activity (Lundström *et al.*, 1992). Recently, it has been suggested that the human gene for PDI can be divided into separate domains, suggesting that PDI is a modular protein (Freedman *et al.*, 1994). Studies comparing PDI with Trx suggest a cellular interaction between PDI, Trx, and TR that may have profound effects on correct protein maturation.

PDI was identified as the β-subunit of prolyl hydroxylase, a key enzyme involved in maturation of collagen (Koivu *et al.*, 1987). Subsequently, the β-subunit for prolyl hydroxylase was cloned and found to encode for PDI (Pihlajaniemi *et al.*, 1987). Prolyl hydroxylase is a heterotetramer (a2b2) that catalyzes the conversion of proline to hydroxyproline in the consensus sequence of Pro-Pro-Gly found in nascent procollagen. The absence of PDI in prolyl hydroxylase leads to an inactive form of the enzyme and blocks secretion of collagen. Part of the function of PDI may be to help prevent the oxidation of collagen leading to a terminally blocked monomer species (Forster and Freedman, 1984).

In addition to a role in correct disulfide bond formation, other functions have been ascribed to PDI. PDI has been suggested to have a chaperonin function in the endoplasmic reticulum (Wang and Tsou, 1993). PDI, in conjunction with GSH and Grx, is able to reduce dehydroascorbate to ascorbic acid, suggesting that these three components may comprise the eukaryotic dehydroascorbate reductase activity (Wells *et al.*, 1990). PDI has been identified as a component in the microsomal triacylglycerol transfer complex, which is required for the assembly and secretion of very low-density lipoproteins and chylomicrons by the liver and intestine, respectively (Wettrau *et al.*, 1991). The presence of PDI in the microsomal triacylglycerol complex is essential for catalytic activity and for preventing aggregation of the other 88 kDa subunit (Wettrau *et al.*, 1991).

2.4. Glutaredoxin

Grx is a 11–12 kDa redox active protein (Wells *et al.*, 1993). Unlike the Trx/TR system, Grx does not have a specific reductase that functions as an electron donor to the oxidized form of Grx. Instead, Grx is reduced through interaction either with GSH and GSH reductase, or with GSH and PDI. The structure of bacterial Grx in the oxidized and reduced state has been investigated by high-field NMR (Xia *et al.*, 1992). Minor differences in structure between the redox states of Grx were found, and Grx had an overall similar folding pattern to bacterial Trx. In contrast to Trx, only Cys-11 of the active site of Grx was exposed on the solvent surface, and Cys-14 was solvent-inaccessible.

Eukaryotic Grx has been purified from yeast (Gan *et al.*, 1990), human placenta (Larson *et al.*, 1985), calf thymus (Luthman and Holmgren, 1977), pig liver (Gan and Wells, 1987), rabbit bone marrow (Hopper *et al.*, 1989), and rat liver (Axelsson *et al.*, 1978). The exact function of eukaryotic Grx remains unclear. For some time, one of the suggested biological functions of Grx was the transfer of electrons to RR (Xia *et al.*, 1992). It has become clear, however, that neither GSH, Grx, (Weckbecker and Cory, 1988) nor Trx (Hopper and Iurlano, 1983) are essential for activation of RR, suggesting the involvement of an additional redox factor.

A biochemical property of Grx that has been identified *in vitro*, and referred to in Section 2.3, is the reduction of dehydroascorbate to ascorbic acid through an interaction with GSH and PDI (Wells *et al.*, 1993). This is an intriguing finding since, despite extensive study, a dehydroascorbate reductase protein has not been purified from animal sources.

2.5. Redox Factor-1

Ref-1 is a 37 kDa nuclear protein originally isolated from HeLa cell nuclear extracts, which catalyses the reduction of the transcription protein complex AP-1 (Abate *et al.*, 1990; Xanthoudakis *et al.*, 1994). Two cysteine residues are critical for the redox activity of Ref-1: Cys-65 and Cys-83 (Walker *et al.*, 1993). There is a direct cysteine-mediated interaction between Ref-1 and Jun, one of the components

of AP-1 (Xanthoudakis *et al.*, 1994). Ref-1 also stimulates DNA binding by other transcription factors, including NF-$\varkappa$B (weakly), Myb, cyclic AMP-responsive element binding protein (Xanthoudakis *et al.*, 1994), and EGR-1 (Huang and Adamson, 1993). Ref-1 can be regenerated by treatment with bacterial reduced Trx, which itself does not reduce AP-1 (Abate *et al.*, 1990), so that the redox sequence in the cell may be NADPH to TR, to Trx, to Ref-1, to AP-1. Ref-1 has also been found to have an apurine/apyrimidine endonuclease DNA repair activity and has a core domain that is highly conserved in a family of prokaryotic and eukaryotic DNA repair enzymes (Xanthoudakis *et al.*, 1992, 1994). The DNA repair activity of Ref-1, however, is separate from its redox activity. Sequences in the *N*-terminal domain of Ref-1 are required for redox activity, while *C*-terminal sequences are necessary for DNA repair activity (Xanthoudakis *et al.*, 1994). Chemical alkylation or oxidation of Ref-1 inhibits its redox activity, but does so without affecting its DNA repair activity (Xanthoudakis *et al.*, 1994). Site directed mutagenesis has identified Cys-65 as the redox active site of Ref-1 (Okuno *et al.*, 1993).

3. REDOX CONTROL OF TRANSCRIPTION FACTOR ACTIVITY

3.1. Nuclear Factor-$\varkappa$B/Rel

NF-$\varkappa$B is a ubiquitous transcription factor that is activated in many cell types in response to growth stimuli and stress, leading to the rapid induction of genes encoding growth and defense proteins (Grimm and Baeuerle, 1993; Thanos and Maniatis, 1995). NF-$\varkappa$B is important for the cellular response to oxidative stress (Meyer *et al.*, 1993) and is a promoter of tumorigenesis (Higgins *et al.*, 1993). NF-$\varkappa$B is a heterodimer composed of p50 and p65 subunits. Activation of NF-$\varkappa$B occurs independently of protein synthesis and involves posttranslational mobilization from an inactive cytoplasmic complex with the inhibitory subunit, I$\varkappa$B. A protein highly related to p65 of NF-$\varkappa$B is the proto-oncogene c-Rel. NF-$\varkappa$B and other members of the v- and c-Rel oncoprotein family have a characteristic cysteine and three arginine residues in the DNA binding region (Ghosh *et al.*, 1990). Several different forms of I$\varkappa$B have been identified, including I$\varkappa$Bα, β, γ, and Bcl-3, and all have multiple ankrin repeats, which mediate their interaction with NF-$\varkappa$B. Upon activation, I$\varkappa$B is cleaved from the complex, and the p50/p65 heterodimer is translocated to the nucleus where p50 binds to the decameric $\varkappa$B motif found in the enhancers of numerous cellular genes. Subtle changes in response are thought to be regulated by different subunits of Rel/NF-$\varkappa$B homo- and heterodimers binding with different affinities to variants of the $\varkappa$B motif. Protein kinase C (PKC), cyclic AMP-dependent kinase, and Raf-1 kinase activate transcription of NF-$\varkappa$B by serine/threonine phosphorylation, leading to the release of the inhibitory I$\varkappa$B-subunit (Yao and O'Dwyer, 1994; Shirakawa and Mizel, 1989). Ras is an activator of Raf-1 kinase, which may explain the ability of Ras to stimulate transcription by NF-$\varkappa$B (Finco and Baldwin, 1993).

NF-$\varkappa$B activity is also redox regulated. Studies by Toledano and Leonard (1991) found that the DNA binding of NF-$\varkappa$B, measured by gel shift assays, is irreversibly inhibited by N-ethylmaleimide (NEM), an alkylator of sulphydryl groups, and reversibly inhibited by diamide, which oxidizes sulphydryl groups. In this study, DTT reversed the effects of diamide, but did not increase the DNA binding of untreated NF-$\varkappa$B. Work by Hayashi *et al.* (1993) also found that the DNA binding of NF-$\varkappa$B was decreased by diamide, and that DTT and 2-mercaptoethanol, but not reduced GSH, increased the DNA binding of NF-$\varkappa$B prepared under anaerobic conditions in the absence of pretreatment with oxidizing agent (Hayashi *et al.*, 1993). The difference in the results between these two studies may be the conditions under which NF-$\varkappa$B was prepared leading to partial oxidation of NF-$\varkappa$B. While reducing conditions do not dissociate the NF-$\varkappa$B/I$\varkappa$B complex, it can be shown that the p50/p65 heterodimer is activated by 2-mercaptoethanol after detergent dissociation of the NF-$\varkappa$B/I$\varkappa$B complex. Matthews *et al.* (1992) studied the DNA binding activity of an NF-$\varkappa$B p50 subunit and found that it was increased by DTT and abolished by NEM. The critical residue in p50 was identified as Cys-62, and its deletion was found to decrease the affinity and specificity of the DNA binding.

Other studies in whole cells have reported an increase in NF-$\varkappa$B binding and transactivation under oxidizing conditions that can be blocked by antioxidants. Staal *et al.* (1990) found that cells stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA) or TNF-α showed a 25% decrease in cellular GSH and an increase in a NF-$\varkappa$B reporter activity. N-acetylcysteine (NAC), a cysteine derivative and a

GSH precursor, protects against TPA- and TNF-α-dependent NF-κB activation (Staal *et al.*, 1990; Shibanuma *et al.*, 1994). Antioxidants such as butylated hydroxyanisole, nordihydroquaretic acid and α-tocopherol also block NF-κB transactivation and function in unstimulated, TPA-, and TNF-α-stimulated cells (Israel *et al.*, 1992). While activation of NF-κB is also inhibited by α-tocopherol acetate and succinate (Suzuki and Packer, 1993a), only α-tocopherol succinate, but not acetate or α-tocopherol, directly inhibited the DNA binding of NF-κB (Suzuki and Packer, 1993b). Exposure of HeLa cells to H_2O_2 was found to increase NF-κB DNA binding and transactivation measured using a reporter construct (Meyer *et al.*, 1993). These effects of H_2O_2 were synergistic, with similar effects produced by TPA. TPA has been suggested to produce its effects on NF-κB by the PKC-dependent promotion of oxidant stress in the cells (Meyer *et al.*, 1993). In cells treated with the PKC inhibitor bryostatin to deplete PKC, TPA had no effect on NF-κB binding and transactivation (Schenk *et al.*, 1994). The stimulatory effects of H_2O_2 on NF-κB transactivation are blocked by the antioxidants pyrrolidine dithiocarbamate (PDTC) and NAC. A possible explanation for inhibition of NF-κB activation by antioxidants in intact cells is that they inhibit the phosphorylation and subsequent release of the IκB inhibitor (Beg *et al.*, 1993). Some antioxidants such as α-tocopherol can directly inhibit PKC (Chatelain *et al.*, 1993; Ozer *et al.*, 1993). PDTC does not block DNA binding of NF-κB in unstimulated cells, but appears to block TPA-stimulated binding (Schenk *et al.*, 1994; Meyer *et al.*, 1993). However, PDTC has been reported not to inhibit PKC activity in whole cells (Meyer *et al.*, 1993).

Trx increases the DNA binding of NF-κB in an NADPH-dependent manner, showing approximately a 500-fold greater potency than nonphysiological reducing agents, such as 2-mercaptoethanol or DTT (Hayashi *et al.*, 1993; Sorachi *et al.*, 1992). Trx is also more effective at increasing the DNA binding of NF-κB than L-cysteine, NAC, or GSH (Galter *et al.*, 1994). Oxidized Trx and GSSG inhibit DNA binding of NF-κB (Galter *et al.*, 1994), while GSH is inactive in restoring the DNA binding of diamide-oxidized NF-κB DNA (Hayashi *et al.*, 1993). Trx only reduces free NF-κB and does not reduce the NF-κB complex, presumably because of hindered access to the p50 subunit in the complex. The redox inactive Cys-31 mutant Trx did not reduce NF-κB. Trx was also found to increase the DNA binding of the NF-κB p50 subunit, but not that of a redox inactive form of p50 (Matthews *et al.*, 1992). In this study, transient transfection of cells with Trx cDNA resulted in a large increase in transactivation by NF-κB measured by a reporter construct. In other studies, transient transfection of HeLa cells with Trx cDNA resulted in a dose-dependent decrease in TPA-stimulated, as well as nonstimulated, NF-κB DNA binding and transactivation (Schenk *et al.*, 1994). Exposure of cells to exogenous Trx also resulted in a concentration-dependent inhibition of TPA-stimulated NF-κB activity (Schenk *et al.*, 1994).

Treatment of cells with BCNU induces the TPA-dependent and -independent activation and nuclear translocation of NF-κB, but inhibits NF-κB DNA binding activity (Galter *et al.*, 1994). Under similar conditions, BCNU induces TPA-dependent and -independent AP-1 activation, but has no effect on AP-1 DNA binding. The effects of BCNU were ascribed to increased intracellular concentrations of GSSG due to inhibition of glutathione reductase (Galter *et al.*, 1994). However, BCNU inhibits other reductases, including TR (Suh *et al.*, 1988), so that the effects could equally well be due to alterations in the intracellular concentration of reduced and oxidized Trx, particularly since DNA binding of NF-κB binding is more sensitive to modulation by Trx than GSH.

3.2. Activator Protein-1

AP-1 is a transcription factor whose activation is a prerequisite to growth factor and TPA-stimulated cell growth. AP-1 is composed of the *jun* and *fos* gene products that form homodimeric (Jun/Jun) or heterodimeric (Jun/Fos) complexes. Activation of AP-1 by TPA is thought to involve activation of a nuclear protein phosphatase, which dephosphorylates and thereby activates preexisting c-*Jun* homodimers (Boyle *et al.*, 1991). DNA binding of the Fos-Jun homodimer (AP-1) is also increased by the reduction of a single conserved cysteine in the DNA binding domain of each of the proteins (Abate *et al.*, 1990; Walker *et al.*, 1993). Fos and Jun proteins that have been mutated so that the cysteine residue is replaced by serine show constitutive DNA binding (Abate *et al.*, 1990). The naturally occurring v-*jun* oncogene has a point mutation that gives a serine instead of a Cys-154 residue (Bohmann *et al.*, 1987). Experimentally replacing Cys-154 with serine in Fos increases its transforming ability

(Lafage-Pochitaloff-Huvale *et al.*, 1987). This mutant Fos, when expressed in chicken fibroblasts, shows an increased AP-1 DNA binding activity that is resistant to treatment with the oxidizing agent diamide (Okuno *et al.*, 1993). From these results, it appears that the level of functional Fos-Jun complexes is normally limited by redox regulation, and that escape from redox control enhances transforming activity.

Antioxidants, such as NAC, PDTC, DTT, and butylated hydroxyanisole, increase unstimulated and TPA-stimulated AP-1 DNA binding and transactivation in cells (Schenk *et al.*, 1994; Meyer *et al.*, 1993), which is associated with an increase in c-*fos* and c-*jun* transcription (Meyer *et al.*, 1993; Schenk *et al.*, 1994). Oxidant stress caused by treatment of cells with BSO or diamide can also stimulate the endogenous and inducible expression of c-*fos* and c-*jun*, and increases AP-1 binding (Bergelson *et al.*, 1994). AP-1 proteins can be reduced by a nuclear redox protein, Ref-1, but apparently not by Trx, or GSH and glutathione reductase. DNA binding of AP-1 is inhibited by oxidized Trx more efficiently than GSSG. In contrast, GSSG is more effective than oxidized Trx in inhibiting NF-$\varkappa$B DNA binding activity (Galter *et al.*, 1994).

3.3. p53

The tumor suppressor protein p53, which has transcription factor activity, recently has been shown to be redox controlled (Hainaut and Milner, 1993). Diamide oxidation of a conserved cysteine, which possibly is involved in metal ion binding, inhibits the DNA binding of p53. The authors of this study suggested that a cellular redox factor may facilitate the reduction of p53.

3.4. $\varkappa$U

The transcription factor $\varkappa$U is a heterodimer composed of p70 and p86 subunits (Zhang and Yaneva, 1993). $\varkappa$U can be regulated through the activity of DNA-dependent protein kinase, and its DNA binding requires reduction of intrinsic cysteine residues mediated, perhaps, by a nuclear factor.

κ (kappa)

3.5. v-Ets

v-Ets is a component of the avian acute leukemia virus 26-derived Gag-v-Myb-v-Ets fusion protein. The binding of v-Ets to DNA requires reducing conditions (Wasylyk and Wasylyk, 1993), and is inhibited by the oxidizing agents diamide and NEM. DNA binding of v-ETS probably involves the conserved cys-394, and is more sensitive than c-Ets to inactivation by oxidizing agents because of the loss of the *C*-terminal sequence in v-Ets (Wasylyk and Wasylyk, 1993).

C (cap)

3.6. Other Transcription Factors

inferior correct?

Other transcription factors that are sensitive to redox modulation are E_2F, which requires DTT for DNA binding activity (Abate *et al.*, 1990), and human transcription factor IIIC, whose DNA binding and transcription is increased by DTT and inhibited by NEM (Cromlish and Roeder, 1989). Specific DNA binding of the zinc finger transcription factor EGR-1 is dependent upon the presence of reducing agents, while Ref-1 reduces EGR-1 and increases its DNA binding (Huang and Adamson, 1993). Treatment of cells with H_2O_2, diethylmaleate or BSO, all of which lower intracellular GSH, results in a decreased binding to DNA of the Sp1 and glucocorticoid receptor transcription factors (Esposito *et al.*, 1995). The DNA binding capacity of the transcription factor upstream stimulatory factor is decreased under nonreducing conditions, and two cysteine residues, both present within the helix-loop-helix protein-protein interface domain, have been identified as the critical sites in this regulation (Pognonec *et al.*, 1992). HoxB5, a sequence-specific DNA binding protein, shows increased DNA binding under oxidizing conditions (Galang and Hauser, 1993). Not all transcription factors are sensitive to redox modulation. DNA binding of serum response factor, a cysteine containing transcription factor that, among other activities, has been implicated in the transcription of c-*fos*, is not sensitive to diamide oxidation or reduction by 2-mercaptoethanol (Tolendano and Leonard, 1991). However, transactivation of a serum response element promoter construct was reported to be increased by PDTC in intact cells (Meyer *et al.*, 1993). The DNA binding of the ubiquitous

transcription factor Oct-1 in nuclear extracts shows no change under oxidizing or reducing conditions (Staal *et al.*, 1990). The DNA binding of the C/EBP transcription factor is not affected by oxidant exposure (Esposito *et al.*, 1995).

4. CELLULAR RESPONSES TO CHANGES IN THE REDOX ENVIRONMENT

Much of the evidence that has been presented previously for a role for redox modulation in the biological activity of enzymes and transcription factors comes from biochemical studies with broken-cell or partly purified preparations. There is also evidence from work using intact cells that changes in the redox environment can lead to alterations in cell growth and programmed cell death. Some of these redox influences on cell growth and death are shown in Fig. 2.

4.1. Growth Stimulation by Thioredoxin

During the search for novel leukemic growth factors, a small molecular weight protein was isolated, cloned and classified as adult T-cell-derived leukemic growth factor (Tagaya *et al.*, 1988, 1989). This protein was found to have nearly complete homology with the predicted amino acid sequence for Trx (Wollman *et al.*, 1988). Since then, it has been determined by a number of groups that adult T-cell-derived leukemic growth factor and Trx are one and the same species, due to a discrepancy in the reported amino acid sequence of human Trx (Tagaya *et al.*, 1989; Deiss and Kimchi, 1991; Gasdaska *et al.*, 1994). Trx increases DNA synthesis in synergy with a number of cytokines, including IL-1, IL-2 (Wakasugi *et al.*, 1990; Yodoi and Tursz, 1991), IL-4 (Darr and Fridovich, 1986) and IL-6 (Ifversen *et al.*, 1993). Trx restores the responsiveness of lymphocytes to phytohemagglutinin in GSH-depleted media (Iwata *et al.*, 1994). Biguet *et al.* (1994) have reported that stimulation of lymphocyte proliferation by *E. coli* Trx is associated with the mobilization of PKC. In addition to stimulating the proliferation of lymphoid cells, human Trx has been shown to stimulate the proliferation of fibroblasts (Oblong *et al.*, 1994a; Wollman *et al.*, 1988) and a variety of human solid tumor cell lines (Nakamura *et al.*, 1992; Powis *et al.*, 1994; Gasdaska *et al.*, 1995). The stimulation of cell proliferation by human Trx is dependent on its reduction and is not seen with a redox-inactive mutant Trx (Oblong *et al.*, 1994a; Gasdaska *et al.*, 1995). *E. coli* Trx stimulates DNA synthesis of lymphoid cells (Biguet *et al.*, 1994), but has no effect on the proliferation of MCF-7 human breast cancer cells (Gasdaska *et al.*, 1995), suggesting that structural features, in addition to the conserved catalytic domain, are necessary for the stimulation of, at least, solid tumor cell proliferation by human Trx.

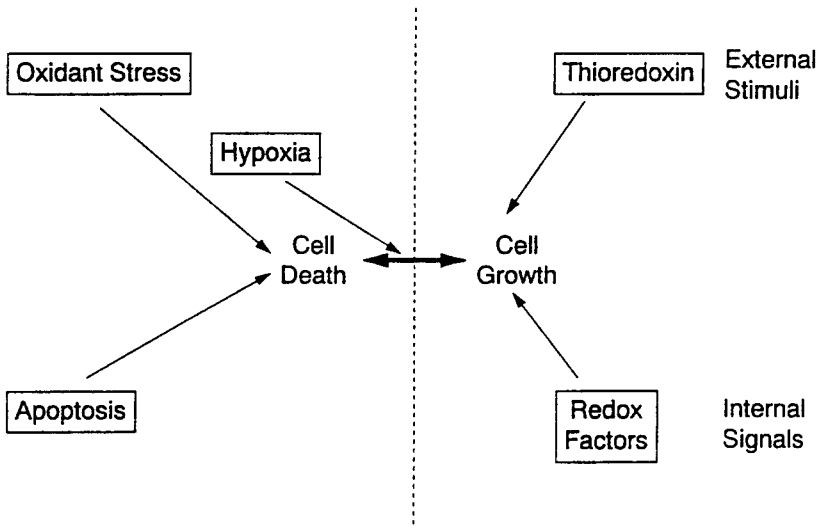

Fig. 2. The effect of redox influences on the balance of cell growth and death. For details, see the text.

There is precedent for Trx-like active-site domains present in growth factors. It has been reported that the β-subunit of human FSH contains a Trx-like active site in the receptor-binding domain (Boniface and Reichert, 1990). Furthermore, using peptides of this domain, it was shown that cell signalling was dependent upon the active-site cysteines, since redox-inactive peptides containing serines could not propagate a signal across the membrane, even though the mutant peptides could bind with the hormone's receptor (Grasso *et al.*, 1993). However, a 14-mer peptide spanning the 8–14 amino acids in the active site of human Trx failed to stimulate cellular proliferation, indicating that structural information, in addition to that of an intact active site, is ~~more than likely~~ required for stimulating proliferation (Oblong *et al.*, 1994b).

Trx mRNA levels are elevated in some human solid lung tumors compared with the paired normal tissue (Gasdaska *et al.*, 1994), while Trx protein levels are increased in human cervical neoplastic squamous epithelial cells (Schallreuter *et al.*, 1990) and hepatocellular carcinoma (Nakamura *et al.*, 1992). Trx can be secreted by cells (Ericson *et al.*, 1992; Rubartelli *et al.*, 1992, 1995) using a nonclassical leaderless pathway (Rubartelli *et al.*, 1992). Thus, it is possible that Trx could be an autocrine growth factor. It is not clear yet if the target of extracellular Trx is a Trx-specific receptor, a surface-bound TR, or another proteinaceous factor requiring reduction of disulfides, or uptake of Trx into the cell and an intracellular action, perhaps on transcription factors, leading to altered gene expression.

4.2. Oxidative Stress

Exposure of cells to H_2O_2 as a model of oxidative stress has multiple effects on redox-regulated activities of the cell. A number of immediate-early genes are transcriptionally activated in cells treated with H_2O_2, including c-*fos*, c-*jun*, and *egr*-1 (Nose *et al.*, 1991; Ohba *et al.*, 1994; Datta *et al.*, 1993; Rao *et al.*, 1993; Amstad *et al.*, 1992). In some cells, there is an increase in inositol(1,4,5)trisphosphate and cytoplasmic-free Ca^{2+} concentration (Schieven *et al.*, 1993b) and an increase in PKC activity (Larsson and Cerutti, 1989). In PC12 cells, H_2O_2 causes a decrease in bradykinin-induced increases in inositol(1,4,5)trisphosphate and cytoplasmic-free Ca^{2+} concentration, apparently mediated by the action of PKC (Klyszcz–Nasko *et al.*, 1993). In lymphoma cells, there is tyrosine phosphorylation of multiple cellular proteins associated with activation of $p72^{Syk}$, but no activation of Src family kinases (Schieven *et al.*, 1993a). Levels of Trx in lymphocytes have also been reported to be increased by H_2O_2 (Hayashi *et al.*, 1993). As noted in Section 3.1, H_2O_2 treatment of cells, apparently acting downstream of PKC, results in activation of NF-κB and potentiation of the stimulation of NF-κB activity by TPA (Meyer *et al.*, 1993). In contrast, the DNA binding of AP-1 is only weakly induced by H_2O_2 (Meyer *et al.*, 1993; Rao *et al.*, 1993; Amstad *et al.*, 1992). However, H_2O_2 and reactive oxygen species (ROS) cause a marked increase in the transcription of c-*jun* as an early response gene (Rao *et al.*, 1993; Amstad *et al.*, 1992). The effect is suppressed by inhibitors of PKC (Amstad *et al.*, 1992) and PLA2 (Rao *et al.*, 1993) and requires poly ADP ribosylation of chromosomal proteins (Amstad *et al.*, 1992). Exposure of epithelial cells to pyrogallol, a generator of ROS, increases the DNA binding of NF-κB, which is followed by an increase in inducible nitric oxide synthase mRNA (Adcock *et al.*, 1994). Naphthoquinone and orthovanadate, both sources of ROS, stimulate protein tyrosine phosphorylation in cells and increase phosphatidylinositol-3-kinase activity and DNA synthesis (Chen and Chan, 1993; Chen *et al.*, 1990). H_2O_2 is produced endogenously in HeLa cells in response to PKC activation (Bhimani *et al.*, 1993). Since exogenous H_2O_2 and superoxide can stimulate the growth of cells, it has been suggested that they may play a role in normal cell growth (Burdon and Gill, 1993).

4.3. Hypoxia

Exposure of HT-29 colon carcinoma cells to hypoxia causes increased transcription of Ref-1 (Yao *et al.*, 1994) and the selective induction of c-*jun* transcription, leading to increased AP-1 binding activity (Yao and O'Dwyer, 1994). The levels of both c-*jun* and *junD* mRNA increase during hypoxia and fall on reoxygenation (Yao *et al.*, 1994). Increases in *fos* mRNA are less pronounced during hypoxia, but are maintained on reoxygenation. During hypoxic exposure of HT-29 cells, increased binding to the AP-1 response element leads to transcriptional induction of the antioxidant enzyme

DT-diaphorase (Yao *et al.*, 1994). There is also an increase in the activity of γ-glutamylcysteine synthetase, the rate-limiting event in GSH synthesis. Nucleosomal degradation, indicative of apoptosis, also increases after a few hours of hypoxia (Yao *et al.*, 1995). Exposure of Jurkat T cells to hypoxic conditions for several hours has been reported to result in an increased NF-κB DNA binding and transactivation due to tyrosine phosphorylation and dissociation of the IκB inhibitory subunit (Koong *et al.*, 1994a). Dominant negative mutants of Ha-Ras and Raf-1 inhibit NF-κB induction, suggesting that it is a downstream event of Ras and Raf-1 kinase activation (Koong *et al.*, 1994b).

4.4. Apoptosis

The cellular redox state appears to play a role in apoptosis, an evolutionarily conserved process for cell removal (for reviews, see Vaux *et al.*, 1994; Williams, 1994; Cohen, 1993; Schwartz and Osborne, 1993). Oxidative stress occurring during apoptosis could result from an increase in the rate of oxidant production or a reduction in the rate at which radical species are removed. Potential targets of oxidative stress during apoptosis include DNA, transcription factors, and proteins that regulate Ca^{2+} homeostasis.

An early report of oxidative stress mediating apoptosis during embryogenesis demonstrated that a H_2O_2-like activity in blastocele fluid causes apoptosis of embryonal carcinoma cells with trophectodermal potential (Pierce *et al.*, 1991). In this case, the increased activity of an amine oxidase is the likely mechanism for the generation of the oxidizing species (Pierce *et al.*, 1990). Cells with embryonic potential do not undergo apoptosis when incubated with blastocele fluid, but lose this resistance if first treated with BSO to deplete GSH (Pierce *et al.*, 1991). The increased production of oxidants appears to underlie apoptosis mediated by TNF-α. This cytokine is a 17.3 kDa protein that was first isolated following the observation of spontaneous tumor regression in cancer patients who contracted bacterial infections (Semenzato, 1990). Purified recombinant TNF-α subsequently was found to be cytotoxic for some tumor lines (Sugarman *et al.*, 1985). Evidence for the importance of oxidative stress to the mechanism of TNF-α-mediated cytotoxicity includes the increased production of hydroxyl radicals with treatment of sensitive, but not resistant, cell lines (Yamauchi *et al.*, 1989), the diminution of cell killing under anaerobic conditions (Matthews *et al.*, 1987) and in respiration-deficient cells (Schulze-Osthoff *et al.*, 1993) and the finding that manganese superoxide dismutase is essential for resistance to the cytokine (Wong *et al.*, 1989). The molecular mechanism by which TNF-α induces a state of oxidative stress remains unclear.

Oxidative stress during apoptosis may be due to a decrease in the cellular antioxidant defenses. This is seen during apoptosis of murine WEHI7.2 lymphocytes, induced by the synthetic glucocorticoid hormone dexamethasone (Briehl *et al.*, 1995). These changes include an early decrease in transcript levels for the antioxidant defense proteins catalase, copper,zinc-superoxide dismutase, ~~manganese superoxide dismutase~~ (MnSOD) and Trx. At the same time, the level of glutathione *S*-transferase mu class mRNA and protein increase. The latter enzyme can function to reverse oxidative damage and is particularly active with products of lipid peroxidation (Berhane *et al.*, 1994). Lipid peroxidation has been detected during dexamethasone-induced apoptosis of a T-cell hybridoma (Hockenbery *et al.*, 1993).

Weakened antioxidant defenses and oxidant stress are seen in disease states characterized by inappropriate cell death. Genetic studies of individuals with Amyotrophic Lateral Sclerosis have identified mutations in the gene coding for copper,zinc-superoxide dismutase (Deng *et al.*, 1993). These mutations result in decreased enzyme activity, which may contribute to the observed pathology of motor neuron death. $CD4^+$ cells and lymph nodes from acquired immunodeficiency syndrome patients have decreased levels of GSH (Staal *et al.*, 1992) and Trx (Masutani *et al.*, 1992), respectively. Catalase, MnSOD, and glutathione peroxidase activities drop in T-cell lines grown *in vitro*, after infection with the human immunodeficiency virus (HIV) (Greenspan and Aruoma, 1994; Sandstrom *et al.*, 1994). The 8E5 HIV-expressing human T-cell line undergoes apoptosis when incubated with hydroperoxy fatty acids, while uninfected cells remain viable (Sandstrom *et al.*, 1994). Hydroperoxy fatty acids are formed by oxidation of cellular membranes and normally metabolized by glutathione peroxidases. Thus, HIV infection appears to weaken the cellular antioxidant defense and increase susceptibility to apoptosis triggered by products of oxidative damage. Studies of the HIV Tat gene, which encodes a transcription factor, suggest that the dampening of the antioxidant defense may

be mediated, at least in part, at the level of gene expression; HeLa cells transfected with the Tat gene exhibit depressed MnSOD gene expression and enzyme activity and increased levels of oxidized proteins (Flores *et al.*, 1993). β-Amyloid is a neurotoxic peptide that aggregates in the brain of Alzheimer's patients and has been found to generate free-radical peptides (Hensley *et al.*, 1995). This has led to the hypothesis that oxidative stress, specifically, membrane damage mediated by the β-amyloid-derived radicals, leads to the neurodegeneration seen with Alzheimer's disease.

The *bcl*-2 oncogene blocks apoptosis in diverse systems and protects cells against oxidative stress-induced damage (Hockenbery *et al.*, 1993) and killing (Kane *et al.*, 1993). *bcl*-2 was first identified in association with the t(14;18) translocation in B-cell lymphomas, which places the Bcl-2 protein coding sequence under the control of an immunoglobulin promoter (for reviews, see McDonnell *et al.*, 1993; Korsmeyer, 1992). The oncogene encodes a 25 kDa integral membrane protein, whose precise function remains unclear. The studies of Hockenbery *et al.* (1993) demonstrate that expression of Bcl-2 in a T-cell hybridoma prevents apoptosis and lipid peroxidation that occur when unprotected cells are treated with glucocorticoids. Kane *et al.* (1993) have shown that Bcl-2 blocks GT1-7 neural cell death brought on by depletion of GSH. In this case, the mode of cell death is not apoptosis, but necrosis, suggesting that Bcl-2 counteracts a cellular process that may lead to either death mechanism. Notably, Bcl-2 expression in the GT1-7 cells resulted in an approximate doubling of the intracellular GSH content. Reoxygenation of HT-29 colon cancer cells after a period of hypoxia leads to a large increase in apoptosis (Yao *et al.*, 1994). There is also an increase in ref-1 mRNA, c-*myc* mRNA, and a late increase in *bcl*-2 mRNA.

Other treatments that supplement or enhance the cellular antioxidant defense increase resistance to apoptosis. NAC, a thiol antioxidant and GSH precursor, blocks apoptosis of spinal ganglion neurons and oligodendrocytes triggered by limiting trophic factors (Mayer and Noble, 1994) and activation-induced apoptosis in T-cell hybridomas (Sandstrom *et al.*, 1995). PDTC, an antioxidant and metal chelator, protects rat thymocytes against dexamethasone- and etoposide-induced apoptosis (Wolfe *et al.*, 1994). Trx protects U937 lymphoma cells against TNF-α-mediated cell killing (Matsuda *et al.*, 1991). The survival of embryonic mouse neurons is enhanced by the thiol compounds Trx, 2-mercaptoethanol, and NAC (Hori *et al.*, 1994). In these same studies, U251 astrocytoma cells were seen to produce increased levels of Trx in response to H_2O_2 treatment. Elevated Trx is also observed in glial cells of the gerbil brain during reperfusion after ischaemia (Tomimoto *et al.*, 1993). Thus, Trx secreted by glial cells may protect neurons, *in vivo*, from oxidative stress-induced cell death.

Conditions that weaken the cellular antioxidant defense increase sensitivity to apoptosis. BSO treatment depletes cells of GSH and sensitizes them to cell killing mediated by γ-irradiation (Dethmers and Meister, 1981) and alkylating agents (Fernandes and Cotter, 1994). In the latter studies, the mode of cell death with the alkylating agents alone and in combination with BSO was seen to shift from apoptosis to necrosis, respectively.

5. CONCLUSION

Redox signalling affords the cell a mechanism whereby it can respond to changes in its external environment through the modulation of the activity of certain genes, ultimately leading to an alteration in cell proliferation or cell death, as circumstances may require. Redox signalling occurs not only in response to changes in external redox conditions, although experimentally this is the way it is most easily investigated, but is a mechanism whereby a cell can also respond to nonredox external stimuli. Changes in a cell's ability to handle oxidative stress can result in either an increased susceptibility or resistance to cell killing, and contribute to several disease processes.

Post-translational redox modification of proteins is more difficult to study than protein phosphorylation. In the latter case, there is a semipermanent modification of the protein with an easily identified phosphate group. Redox modification has no such convenient marker, and is readily reversed when the intracellular environment becomes exposed to the extracellular oxidizing environment. Conversely, the use of chemical reducing agents in biochemical systems to mimic the intracellular reducing environment may serve to mask the changes in protein activity that can occur through subtle redox changes. For this reason, appreciation of the potential importance of intracellular redox signalling has lagged behind that of other signalling mechanisms in the cell.

An analogy can be made between redox signalling and changes in intracellular free Ca^{2+} as signalling mechanisms. The intracellular free Ca^{2+} concentration is tightly regulated at very low levels in the face of a high extracellular Ca^{2+} concentration. Small, transient changes in the intracellular free Ca^{2+} concentration are used by the cell as a signalling mechanism to regulate a variety of cell functions (Villereal and Byron, 1992). In a similar manner, cells maintain a highly reduced intracellular environment in the face of oxidizing external conditions. Small and, probably, transient changes in the redox state of key intracellular proteins provide the cell with another important signalling mechanism.

To function as a true signalling cascade, there must be amplification of the original signal. The transfer of reducing equivalents is a stoichiometric process that, in itself, cannot lead to amplification. However, activation of enzymes that regulate the transfer of reducing equivalents from small molecules in the cell, such as NADH, NADPH, and GSH, can lead to signal amplification. While many of the latter steps of the redox regulation of protein activity are being understood, particularly the specificity that can occur in such reactions, the early steps involving regulation of reducing enzymes that can lead to signal amplification remains to be elucidated.

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1. "Hockenberry" or "Hockenbery" as in references?

2. Update? done

Decreased Antioxidant Defense and Increased Oxidant Stress During Dexamethasone-Induced Apoptosis: *bcl-2* Selectively Prevents the Loss of Catalase Activity

Running Title: *bcl-2* stabilizes catalase activity

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Abstract

When the WEHI7.2 mouse lymphoid cell line was treated with dexamethasone to induce apoptosis the activities and transcript levels of the antioxidant defense enzymes catalase, superoxide dismutase (SOD) and DT-diaphorase exhibited a progressive decrease over 48 hours. Catalase activity was selectively maintained following dexamethasone treatment of WEHI7.2 cells transfected with the *bcl-2* oncogene, which protects the cells against apoptosis. Treatment of wild-type WEHI7.2 and *bcl-2* transfected cells with a catalase inhibitor, aminotriazole, was not sufficient to induce apoptosis. Antioxidants, including bovine liver catalase, bovine erythrocyte CuZnSOD, sodium selenite and Trolox, a water soluble vitamin E analog, as well as hypoxia, inhibited dexamethasone-induced apoptosis. These results suggest that oxidant stress due to the decreased activity of antioxidant defense enzymes may play a role in dexamethasone-mediated lymphoid cell apoptosis and that *bcl-2* may prevent apoptosis by maintaining the level of critical antioxidant defense mechanisms, which include catalase.

Keywords

apoptosis, lymphocytes, *bcl-2*, antioxidant enzymes, oxidative stress

Abbreviations

The abbreviations used are: CuZnSOD, copper-zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; GPX, glutathione peroxidase; PBS, phosphate-buffered saline; GST, glutathione S-transferase; aminotriazole, 3-amino-1,2,4-triazole; Trolox, 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid.

Introduction

Apoptosis is a physiological form of cell death that plays a role in tissue homeostasis. The morphological features of apoptosis are seen in a range of cell types in response to diverse stimuli. The shared morphological changes and apparent need for timely, precise regulation of cell death in multicellular organisms has led to the concept of a common, underlying mechanism for cell removal, which is subject to genetic control (Kerr *et al.*, 1972; Cohen, 1993).

The *bcl-2* gene, which encodes a 25 Kd integral membrane protein, was first identified as a gene that protects malignant B cells against normal cell death and is the prototype of a family of genes that inhibit apoptosis (Korsmeyer, 1992). The protective effect of Bcl-2 in different systems supports the idea of a common mechanism for apoptosis in which Bcl-2 exerts an effect late in the pathway (Reed, 1994). Kane *et al.* (1993) have shown that in neural cells Bcl-2 blocks cell death brought on by depletion of glutathione, a ubiquitous low molecular weight compound that functions to scavenge radical species and reverse oxidative

damage. Hockenberry *et al.* (1993) have shown that expression of Bcl-2 in a T-cell hybridoma prevents oxidative damage that occurs following dexamethasone treatment of non-transfected cells. The ability of Bcl-2 to counteract oxidant stress suggests that apoptosis might be mediated through an oxidant process.

Oxidant stress occurs in cells when there is an imbalance between the rate of generation of oxidizing species and the rate of removal of oxidizing species (Cerutti, 1985). Oxidant stress is known to be toxic to cells (Cerutti, 1985) and, thus, might be a contributing factor to apoptosis. Physiological mediators of oxidative stress can work either by modulating oxidant formation or cellular defenses against oxidizing species. Antioxidant defense enzymes include copper-zinc superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD), which are localized to the cytoplasm and mitochondria, respectively, and which convert the superoxide anion radical to H_2O_2; and catalase and glutathione peroxidase (GPX), which reduce H_2O_2 to water (Forman and Fisher, 1981). DT-diaphorase (NAD(P)H:quinone(acceptor) oxidoreductase) catalyzes the two electron reduction of quinones and other oxidizing species and may also afford protection against oxidant stress (Li and Jaiswal, 1992).

Dexamethasone-induced apoptosis of the thymoma-derived murine WEHI7.2 cell line has been used as a model for T-cell selection occurring *in vivo* (Flomerfelt *et al.*, 1993). Previously, we reported an early decline in the transcript levels for catalase, CuZnSOD, MnSOD and DT-diaphorase during dexamethasone-induced apoptosis in WEHI7.2 cells (Briehl *et al.*, 1995). We proposed that oxidant stress

may result from the dexamethasone-induced down-regulation of cellular antioxidant defense. We now report studies comparing changes in the activities of antioxidant defense enzymes and the effects of antioxidants on apoptosis, following dexamethasone treatment of wild-type and *bcl-2* transfected WEHI7.2 cells.

Results

Measurement of Apoptosis

Dexamethasone treatment of WEHI7.2 mouse lymphoid cells is known to cause a decrease in cell viability and an increase in DNA laddering, characteristic of apoptosis, within 24 h of the addition of hormone (Briehl *et al.*, 1995; Lam *et al.*, 1994). DNA laddering is not, however, a quantitative measurement of apoptosis. We compared two methods that can be used for quantifying apoptosis, both of which are based on DNA fragmentation: an alkaline elution assay for DNA single strand breaks and a commercially available immunoassay for histone-associated DNA fragments (Figure 1). Both techniques exhibited a time course for DNA damage that correlated closely with the time course of DNA laddering seen previously with dexamethasone-treated WEHI7.2 cells (Briehl *et al.*, 1995). WEHI7.2 cells which have been transfected with human *bcl-2* and stably express the Bcl-2 protein (W.HB12 cells) (Lam *et al.*, 1994) exhibited no DNA damage as measured by either the alkaline elution or immunoassay methods. Thus, both techniques appear to be valid for quantifying apoptosis in the WEHI7.2 system. In all further studies apoptosis was measured by the immunoassay-based technique.

Antioxidant enzyme gene expression

In our previous studies with WEHI7.2 cells the transcript levels of a number of antioxidant enzymes were seen to decrease within 8 h of dexamethasone treatment (Briehl *et al.*, 1995). Since DNA fragmentation and laddering occur only after 24 h of dexamethasone treatment, we interpreted the change in antioxidant gene expression as an early, apoptosis-associated event. We have now used Northern blot hybridization analyses to determine whether Bcl-2 prevents the changes in antioxidant enzyme gene expression following dexamethasone treatment. As shown in Figure 2, decreased transcript levels for CuZnSOD, MnSOD, GPX, catalase and DT-diaphorase occurred following dexamethasone treatment of W.Hb12 cells, similar to what was seen for the WEHI7.2 cells. Neither WEHI7.2 nor W.HB12 cells expressed detectable levels of mRNA for metallothionine, another putative antioxidant protein (Dr. J. Lazo, personal communication). The decrease in antioxidant enzyme mRNA levels does not appear to be a generalized decrease in transcription, since levels of ß-actin, histone H3, glyceraldehyde 3-phosphate dehydrogenase and cathepsin D mRNAs did not decrease (results not shown) and the levels of glutathione-S-transferase mRNA actually increased (Figure 2). Glutathione S-transferases conjugate glutathione to electrophilic substrates that include oxidized proteins, lipids and nucleotides, and act to repair oxidant damage in cells (Forman and Fisher, 1981; Berhane *et al.*, 1994).

Antioxidant enzyme activity

We next examined whether the observed changes in antioxidant enzyme transcript levels lead to decreased enzyme activity (Figure 3). Total superoxide dismutase activity (contributed by MnSOD and CuZnSOD) after 48 h of dexamethasone treatment was decreased (± SE) by 73.0 ± 2.1% and 52.6 ± 3.6% ($p < 0.05$) in WEHI7.2 and W.HB12 cells, respectively. At the same time, DT-diaphorase activity was decreased by 50.1 ± 11.0% and 55.9 ± 2.2% ($p > 0.05$) in WEHI7.2 and W.HB12 cells, respectively. Dexamethasone treatment of the WEHI7.2 cells resulted in a 56.7 ± 2.5% decrease in catalase enzyme activity at 48 h, compared to non treated cells, but no significant decrease in catalase activity was seen in the Bcl-2 transfected cells. GPX activity was not detectable in either cell type using an assay with a lower limit of detectability of 1.0 nmol/min/mg (results not shown).

Catalase inhibition

A possible interpretation of the selective protection afforded by *bcl-2* against the loss of catalase activity is that the decrease in catalase activity in dexamethasone-treated WEHI7.2 cells triggers apoptosis. To explore this possibility, we tested whether drug-induced inhibition of catalase activity in WEHI7.2 and W.Hb12 cells resulted in apoptosis. Treatment of the WEHI7.2 and W.Hb12 cells for 38 h with 5 mM aminotriazole, an irreversible catalase inhibitor (Darr and Fridovich, 1986), resulted in a 91.0 ± 3.4% (S.E.) ($p < 0.05$) inhibition of catalase activity (Figure 4). However, no increase in apoptosis was observed in either cell line. These

results suggests that a decrease in catalase activity alone is not sufficient to cause apoptosis. An unexplained observation is that when combined with dexamethasone treatment for 38 h, aminotriazole was markedly less effective at inhibiting catalase activity.

Protection by antioxidants

To further explore the role of oxidative stress during dexamethasone-induced apoptosis of WEHI7.2 cells we tested for the protective effect of different antioxidants. Addition of bovine liver catalase to the culture media inhibited dexamethasone-induced apoptosis by 68.1% (Figure 5). Trolox, a water soluble vitamin E analog, protected against apoptosis by 54.5% and bovine erythrocyte CuZnSOD protected by 28.3%. Bovine erythrocyte GPX and human recombinant thioredoxin had no protective effect. Pretreatment of WEHI7.2 cells for nine days with 100 nM sodium selenite produced a 48.2% decrease in apoptosis following dexamethasone treatment. Sodium selenite treatment also caused the levels of glutathione peroxidase activity to increase from undetectable to 39.3 ± 5.3 nmol NADPH/min/mg protein (S.E.) by 9 days of treatment. The antioxidants N-acetyl-cysteine and pyrrolidine dithiocarbamate have been reported by other investigators to block apoptosis in some systems (Paglia and Valentine, 1967; Wolfe *et al.*, 1994). Treatment of WEHI7.2 cells with comparable concentrations of these antioxidants resulted in appreciable cytotoxicity by 24 h. Thus, these antioxidants could not be used to study protection against dexamethasone-induced apoptosis in the WEHI7.2 system. When oxygen was removed prior to dexamethasone addition

and anaerobic conditions maintained during treatment of WEHI7.2 cells, apoptosis was almost completely abolished. Nucleosomal enrichment factors (± S.E.) after 38 h were 4.94 ± 0.17 and 1.43 ± 0.06 ($p < 0.01$), under euoxic and hypoxic conditions, respectively. Hypoxia for this length of time, itself, had no effect on apoptosis with a nucleosomal enrichment factor of 1.35 ± 0.04 compared to 1.35 ± 0.02 ($p > 0.05$) in euoxic cells.

Discussion

Several lines of evidence support a role for oxidant stress as a mediator of apoptosis. Ionizing radiation, which generates reactive oxygen species, and H_2O_2 treatment can cause apoptosis in mouse lymphoid and myeloid cells (Radford *et al.*, 1994; Lennon *et al.*, 1991). A number of antioxidants have been found to block apoptosis. These include N-acetylcysteine, a thiol antioxidant and glutathione precursor (Dröge *et al.*, 1992) that blocks activation-induced apoptosis in T-cell hybridomas (Sandstrom *et al.*, 1995) and pyrrolidine dithiocarbamate, a free radical scavenger and metal chelator that protects rat thymocytes against dexamethasone and etoposide-induced apoptosis (Wolfe *et al.*, 1994). Increased levels of oxygen radicals are seen in cells treated with tumor necrosis factor-α, a physiological inducer of apoptosis (Larrick and Wright, 1990; Matthews *et al.*, 1987). Finally, the *bcl-2* oncogene, which is able to block apoptosis in diverse systems, protects cells against oxidant stress-induced damage (Hockenberry *et al.*, 1993) and cell killing (Kane *et al.*, 1993; Berhane *et al.*, 1994).

Previously, we reported that dexamethasone treatment of WEHI7.2 cells results in a decrease in transcript levels of a number of antioxidant enzymes, including CuZnSOD, MnSOD, catalase and DT-diaphorase. We now report on corresponding decreases in enzyme activity that occurred as early as 12 h after dexamethasone treatment and preceded apoptosis, which is first seen at 24 h and was maximal at 36 to 48 h. We also observed a decrease in transcript levels for GPX but were not able to detect GPX enzyme activity in WEHI7.2 cells. Similar failure to detect GPX activity, despite measurable transcript levels, has been made in other cell types (Buckman *et al.*, 1993). This may be because of a limited concentration of selenium in the culture medium, which is essential for biological activity of the major form of GPX (Roveri *et al.*, 1994). GPX activity could be increased to detectable levels by growing the WEHI7.2 cells in medium supplemented with selenium, and these cells appeared to be more resistant to dexamethasone-induced apoptosis.

Decreasing the cellular antioxidant defense should make the cell vulnerable to endogenously-generated oxidants. It has been estimated that 2% of the oxygen consumed in cells leaks from electron transport chains in the form of reactive oxygen species (Boveris and Chance, 1973). An important recent finding is that a mutation on the gene on chromosome 21 that codes for CuZnSOD is associated with the slow, progressive degeneration of motor neurons in amyotrophic lateral sclerosis (ALS) (Rosen *et al.*, 1993; Rosen, 1993). This degeneration appears to be due to decreased SOD activity and increased oxidant damage to neuronal tissue of

patients with ALS (Rothstein *et al.*, 1994). The neuronal degeneration of ALS could be a form of oxidant-stress induced apoptosis.

Further evidence that dexamethasone-treated WEHI7.2 cells are exposed to oxidant stress is the finding that catalase, CuZnSOD, Trolox, a water soluble vitamin E analog, and selenium which has antioxidant properties (Shen *et al.*, 1994), added to the culture medium protected the cells against dexamethasone-induced apoptosis. Sandstrom and Buttke (1993) reported the isolation of a protein from the conditioned medium of human CCRF-CEM T-cells that protects the cells against apoptosis in serum-free medium. The protein was identified as catalase. They also found that addition of human erythrocyte or bovine liver catalase to the medium afforded CCRF-CEM cells protection against apoptosis. It has been have reported that transfection of murine FL5.12 cells with GPX, but not MnSOD, cDNA will protect the cells against apoptosis triggered by IL-3 withdrawal (Hockenberry *et al.*, 1993). Others have found that added thioredoxin will protect U937 human lymphoma cells against apoptosis (Matsuda *et al.*, 1991). However, we saw no protection of apoptosis in WEHI7.2 cells when thioredoxin was added to the culture media. The difference may be due to the cell lines studied. We found that hypoxia during exposure of WEHI7.2 cells to dexamethasone almost completely protected the cells against apoptosis. A recent report by Muschel *et al.* (1995) claims to have found no protective effect by hypoxia against dexamethasone-induced apoptosis in the related WEHI7.1 clone. However, the study has several unusual features that make the results difficult to interpret. First, the level of apoptosis induced by dexamethasone in the WEHI7.1 cells was low, only 3 fold at

24 h and involved only 5.5% of the total cell number. Second, the cells were grown at very high density which may be why the hypoxic treatment itself was toxic causing over half the cells to exhibit apoptosis. Our results using WEHI7.2 cells, where dexamethasone-induced apoptosis involves the majority of the cells (Lam *et al.*, 1994), and hypoxic treatment was not toxic clearly suggest that oxygen species may be involved in dexamethasone-induced apoptosis in WEHI7.2 cells.

The protective effect of Bcl-2 appears to be at a late step in the cell death pathway. We observed downregulation of some antioxidant enzyme activities in *bcl-2*-transfected W.Hb12 cells, although SOD activity decreased to a significantly lesser extent than for the nontransfected WEHI7.2 cells. Most notably we found that *bcl-2* expression stabilizes catalase activity over 48 h of dexamethasone treatment, despite falling transcript levels. This is especially intriguing in light of the finding, noted previously, that catalase added to the culture medium protects WEHI7.2 cells against dexamethasone-induced apoptosis. Catalase functions as an antioxidant to protect cells from H_2O_2, a relatively stable molecule that freely crosses cellular membranes and has the potential for inflicting great cellular damage. While it cannot react with membranes directly, H_2O_2 does cooperate with the superoxide anion radical in metal-catalyzed reactions yielding the highly membrane-reactive hydroxyl radical (Halliwell and Chirico, 1993). Since inhibiting catalase with aminotriazole did not cause apoptosis, the loss of catalase activity, alone, is not sufficient to induce apoptosis.

Recent evidence suggests that the cytoplasmic fraction of the cell may contain all the information necessary for the morphological changes of apoptosis. Specifically, enucleated and nucleated human fibroblasts treated with staurosporine were found to exhibit the same cell shrinkage, vacuolation and increased electron density indicative of apoptosis (Jacobsen *et al.*, 1994). Tumor necrosis factor-α can also activate apoptosis in mammalian hematopoietic cells by a mechanism that does not involve the nucleus (Hopkin, 1995). Although dexamethasone-induced apoptosis is a steroid hormone receptor-mediated response that clearly requires changes in gene transcription (Miesfeld, 1989), the final steps leading to apoptosis could still reside in the cytoplasm. Our observations of decreased antioxidant enzyme gene transcription and decreased cytoplasmic antioxidant defense fits the hypothesis that dexamethasone-induced apoptosis requires an initial nuclear gene transcription step, followed by an extranuclear step, in this case oxidative damage, leading to apoptosis.

Materials and Methods

Materials

[2-^{14}C]thymidine (55 mci/mmol) was purchased from Research Products International Corporation (Mount Prospect, IL). Proteinase K was obtained from Fisher Scientific (Pittsburgh, PA). Trolox was purchased from Aldrich Chem Co. (Milwaukee, MI). All other chemical reagents were from Sigma Chemical Co. (St. Louis, MO). Plasmids used to prepare cDNAs of human catalase, MnSOD and

CuZnSOD, rat glutathione-S-transferase and DT-diaphorase, and mouse ß-actin have been described previously (Briehl *et al.*, 1995). The plasmid used to prepare mouse GPX cDNA was a gift from Dr. C. Roland Wolf (Imperial Cancer Research Center, Dundee, U.K.).

Cell culture

The WEHI7.2 parental cell line (Harris *et al.*, 1973) and a *bcl-2* transfected clone, W.Hb12 (Lam *et al.*, 1994) were obtained from Dr. Roger Miesfeld (University of Arizona, Tucson) and tested to be mycoplasma-free. Cells were grown in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY) supplemented with 10% calf bovine serum (Hyclone Laboratories, Logan, UT). Stock cell cultures were kept in log phase growth, 0.05 to 2 x 10^6 cells/ml, at 37°C in a humidified incubator with 6% CO_2. Vehicle alone (0.01% ethanol) or dexamethasone (1 μM) was added to cell cultures at densities in the range of 1 to 5 x 10^5 cells/ml. Hypoxic conditions were achieved by flushing flasks containing cells in medium buffered with 25 mM HEPES, pH 7.4, for 3 h at 37°C with humidified N_2 that was purged of O_2 using a Supelpure-O_2 trap (Supelco Inc., Bellefonte, PA). Vehicle or dexamethasone was added to the flasks which were then sealed for the remainder of the study.

Apoptosis

Apoptosis was measured in two ways. Histone-associated DNA fragments were detected using an ELISA kit (Boehringer Mannheim, Indianapolis, IN) that measures the enrichment of mono- and oligo-nucleosomes in the cytoplasmic fraction of lysed cells (Leist *et al.*, 1994). Quantitative values were calculated as the ratio of absorbance with dexamethasone-treated cells to vehicle-treated control cells. Single strand DNA breaks were measured by alkaline elution as described by Kohn *et al.* (1981). Briefly, cells in logarithmic growth were treated with 0.1 μCi/ml of [2-^{14}C]thymidine for 48 h before treating with 1μM dexamethasone for up to 48 h. Cells (10^6) were loaded onto 2.0 μM polyvinylchloride filters and washed twice with ice cold phosphate-buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4). The cells were immediately lysed under protected UV light with 5 ml of 2% SDS in 0.1 M glycine buffer, pH 10, 20 mM EDTA and 0.5 mg/ml proteinase K. Elution was performed at a flow rate of 1 ml/min over 15 h using a solution of 1% SDS and 20 mM EDTA (buffered with 40% tetrapropyl ammonium hydroxide to pH 12.1). Fractions, 2.5 ml, were collected by suction of the elution fluid through the filter for 15 hours. These fractions were mixed with 7.5 ml of scintillation fluid and radioactivity measured by scintillation counting. Quantitative results were expressed as radiation-equivalents as described by Kohn (1981).

Northern blots

Procedures for the isolation of WEHI7.2 cell total RNA and preparation of Northern blots have been described previously (Briehl *et al.*, 1995). Probes were made by random labelling of purified, cloned cDNA fragments using a DNA labelling kit (Gibco BRL, Gaithersburg, MD). Unincorporated nucleotides were removed using NucTrap push columns (Stratagene, La Jolla, CA). Blots were prehybridized with 0.5 M sodium phosphate, pH 7.2, 10 mM EDTA, 7% SDS and 1% bovine serum albumin for a minimum of 1 h at 65°C. Probes were added to an activity of 10^6 cpm/ml and incubated at 65°C for at least 16 h. Blots were washed twice with 300 mM NaCl, 30 mM sodium citrate, pH 7.0, for 5 min at room temperature and twice in 250 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA and 2% SDS at 65°C for 10 min. Blots were stripped and reprobed with ß-actin cDNA for normalization of loading and transfer. Transcript levels were quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Enzyme assays

After treatment, cells were washed twice with cold PBS. To measure SOD activity cells were centrifuged at 800 x g for 5 min at 4°C and the pellet was resuspended in 100 mM triethanolamine-diethanolamine buffer, pH 7.4. The pellet was homogenized with 10 strokes a teflon homogenizer, sonicated for 10 s using a microtip probe (Heat Systems, Farmington, CT) and then homogenized again with 6 strokes. The lysates were centrifuged at 105,000 x g for 1 h at 4°C and the

supernatant fractions passed through Sephadex G25 spin columns (Pharmacia, Piscatway, NJ). SOD activity was measured by a spectrophotometric method based on the inhibition of superoxide-dependant NADH oxidation as described by Paoletti *et al.* (1986). For measurement of catalase and DT-diaphorase activities, cells were washed in PBS and resuspended in 10 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 0.5 mM DL-dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. Triton X-100 was added to a final concentration of 1% and the samples were incubated for 30 min at 4°C. After centrifugation at 3,000 x g for 15 min, the supernatant fractions were assayed for enzyme activity. Catalase activity was measured as the decrease in the absorbance of H_2O_2 at 240 nm as described by Lu *et al.* (1993). DT-diaphorase activity was measured as the NADPH-dependent reduction of dichlorophenolindophenol at 600 nm that was inhibited by 50 μM dicumarol (Gasdaska *et al.*, 1993). For the measurement of GPX activity, cells were washed twice with PBS and resuspended in 0.25 M sucrose, 5 mM HEPES buffer, pH 7.4, 1 mM EDTA, and sonicated for 90 seconds at 4°C. Cell lysates were centrifuged at 16,500 x g for 15 min at 4°C. The supernatant fractions were then centrifuged at 105,000 x g for 20 min at 4°C, and the cytosolic supernatants were assayed immediately for GPX activity as described by Paglia and Valentine (1967). All enzyme activities were normalized to protein content, determined by the method of Peterson (1977) with bovine serum albumin as the standard.

Figure Legends

Figure 1 Measurement of DNA damage indicative of apoptosis. WEHI7.2 lymphoid cells (■) and *bcl-2* transfected cells (□) were treated with 1 μM dexamethasone or vehicle (0.01% ethanol) before assaying for DNA damage. **A** DNA single strand breaks measured by alkaline elution and **B** histone-associated DNA fragments measured in an ELISA. Bars are standard deviation (n = 15). Each ELISA was conducted in duplicate and repeated three times with similar results.

Figure 2 Expression of antioxidant defense genes during dexamethasone-induced apoptosis. Results from Northern blot hybridization analyses of total RNA extracted from WEHI7.2 (■), and *bcl-2* transfected W.Hb12 cells (□), treated with 1 μM dexamethasone for 0, 12, or 24 h. Blots were probed with ^{32}P-labeled cDNA to: **A** MnSOD, **B** CuZnSOD, **C** GPX, **D** glutathione-S-transferase, **E** DT-diaphorase and **F** catalase. Blots were stripped and reprobed with ^{32}P-labeled ß-actin cDNA sequences. Quantitation was by phosphorimage analyses. Values are expressed relative to non-dexamethasone treated cells and normalized to ß-actin. Values are typical of 1 to 3 experiments.

Figure 3 Antioxidant enzyme activity in dexamethasone-treated lymphocytes. **A** DT-diaphorase, **B** total SOD and **C** catalase activity in cell lysates from WEHI7.2 (●) and *bcl-2* transfected W.Hb12 cells (▽) after treatment with 1 μM dexamethasone for 0, 12, 24, 36, or 48 h. Values are the mean of at least 3 determinations and bars are S.E.

Figure 4 Measurements of enzyme activity and apoptosis after treatment with the catalase inhibitor aminotriazole. **A** Apoptosis, and **B** catalase activity in WEHI7.2 (■) and W.Hb12 (□) cells after treatment with 5 mM aminotriazole (AT) or dexamethasone (Dex) for 38 h. Apoptosis was measured as in **Figure 1B.** Values are the mean of 3 determinations and bars are S.E.

Figure 5 Effect of antioxidants on dexamethasone-induced apoptosis. WEHI7.2 cells were treated for 36 h with vehicle (0.01% ethanol), 1 μM dexamethasone or 1 μM dexamethasone plus 2 μM human recombinant thioredoxin; 4.5 U/ml bovine erythrocyte glutathione peroxidase; 300 U/ml bovine liver catalase; 300 U/ml bovine erythrocyte CuZnSOD; or 10 mM Trolox. Some cells were grown in 100 nM sodium selenite-supplemented media for 9 days before adding 1 μM dexamethasone. Apoptosis was measured as in **Figure 1B.** Values are representative of the mean of at least 3 determinations and bars are S.E.
*$p < 0.05$ compared to dexamethasone-treated cells.

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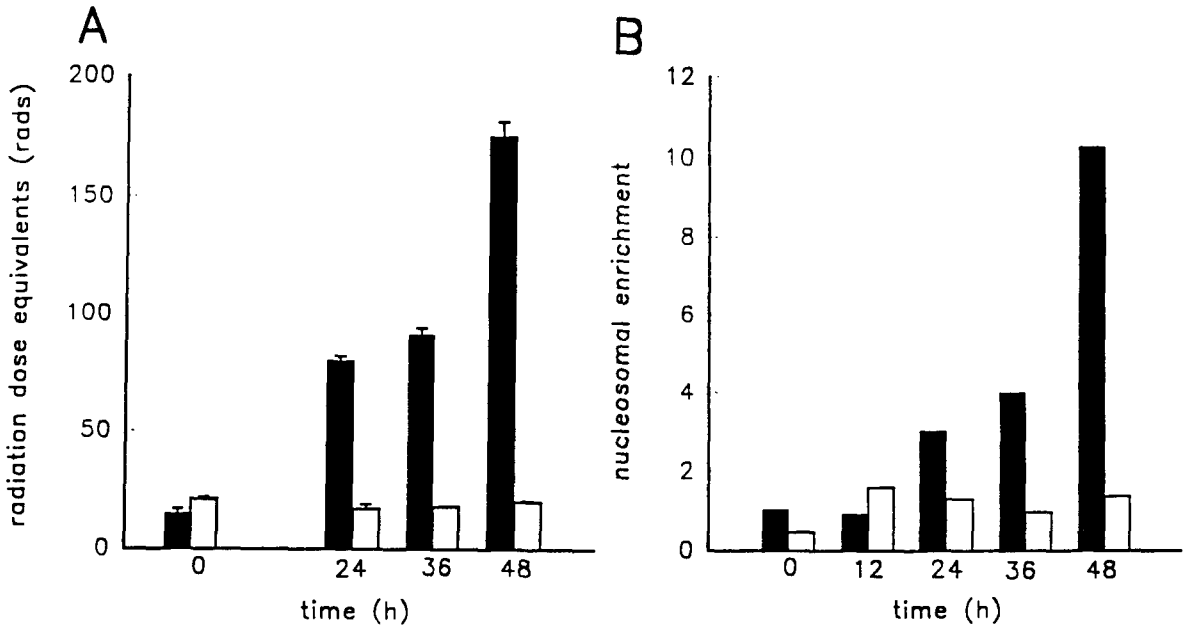
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radiation dose equivalents (rads)
200
150
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time (h)
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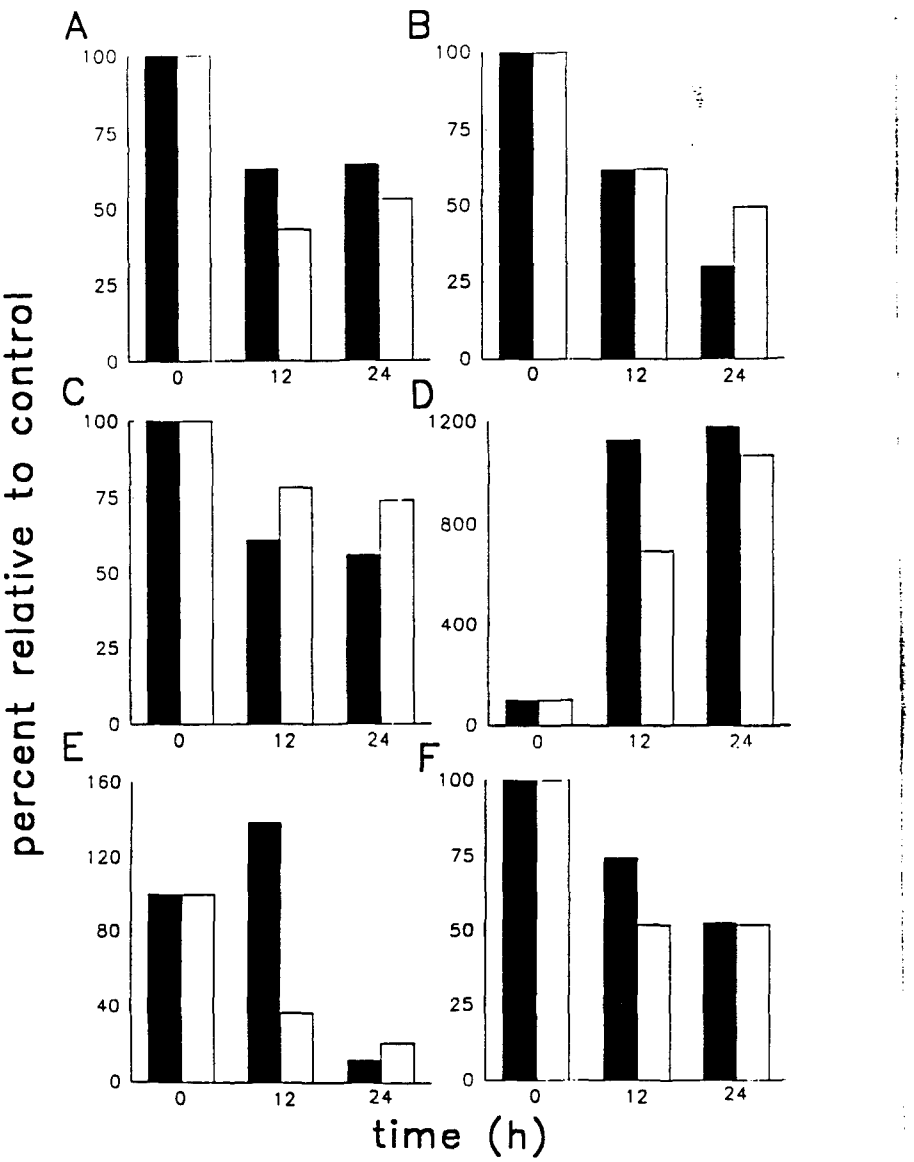
time (h)

Figure 1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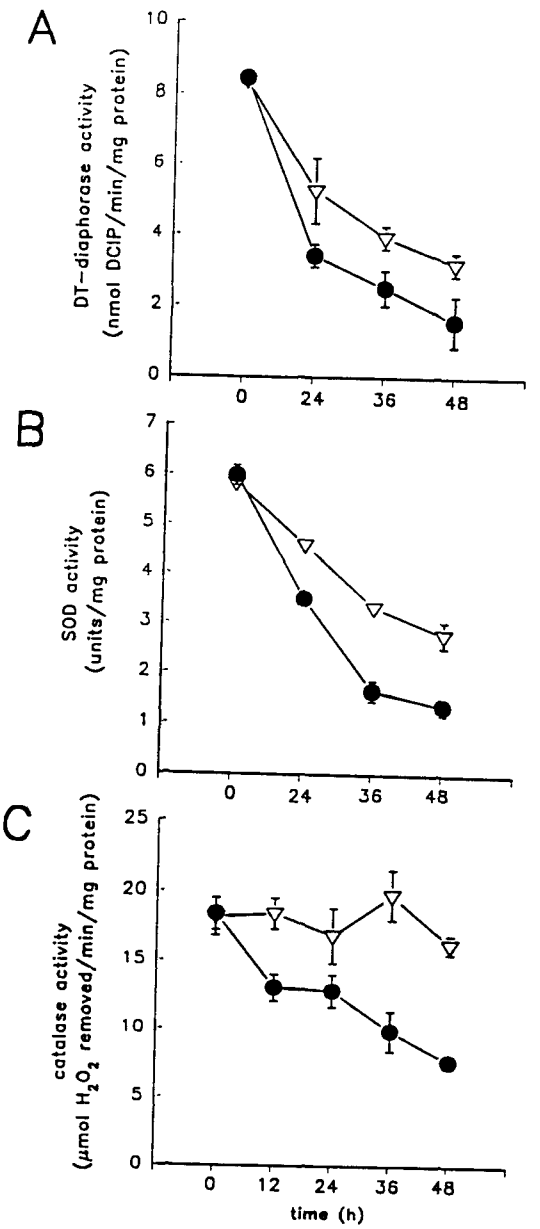
percent relative to control
time (h)

Figure 2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DT-diaphorase activity
(nmol DCIP/min/mg protein)
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8
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2
0
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24
36
48
B
SOD activity
(units/mg protein)
7
6
5
4
3
2
1
0
0
24
36
48
C
catalase activity
(μmol H_2O_2 removed/min/mg protein)
25
20
15
10
5
0
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12
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36
48
time (h)

Figure 3

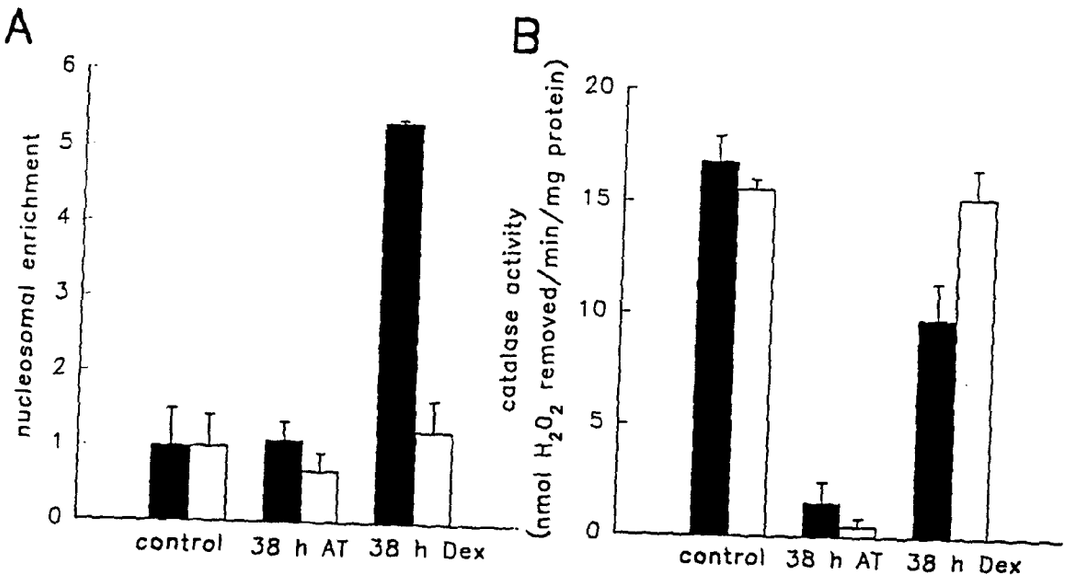
A
nucleosomal enrichment
0
1
2
3
4
5
6
control
38 h AT
38 h Dex
B
catalase activity
(nmol H_2O_2 removed/min/mg protein)
0
5
10
15
20
control
38 h AT
38 h Dex

Figure 4

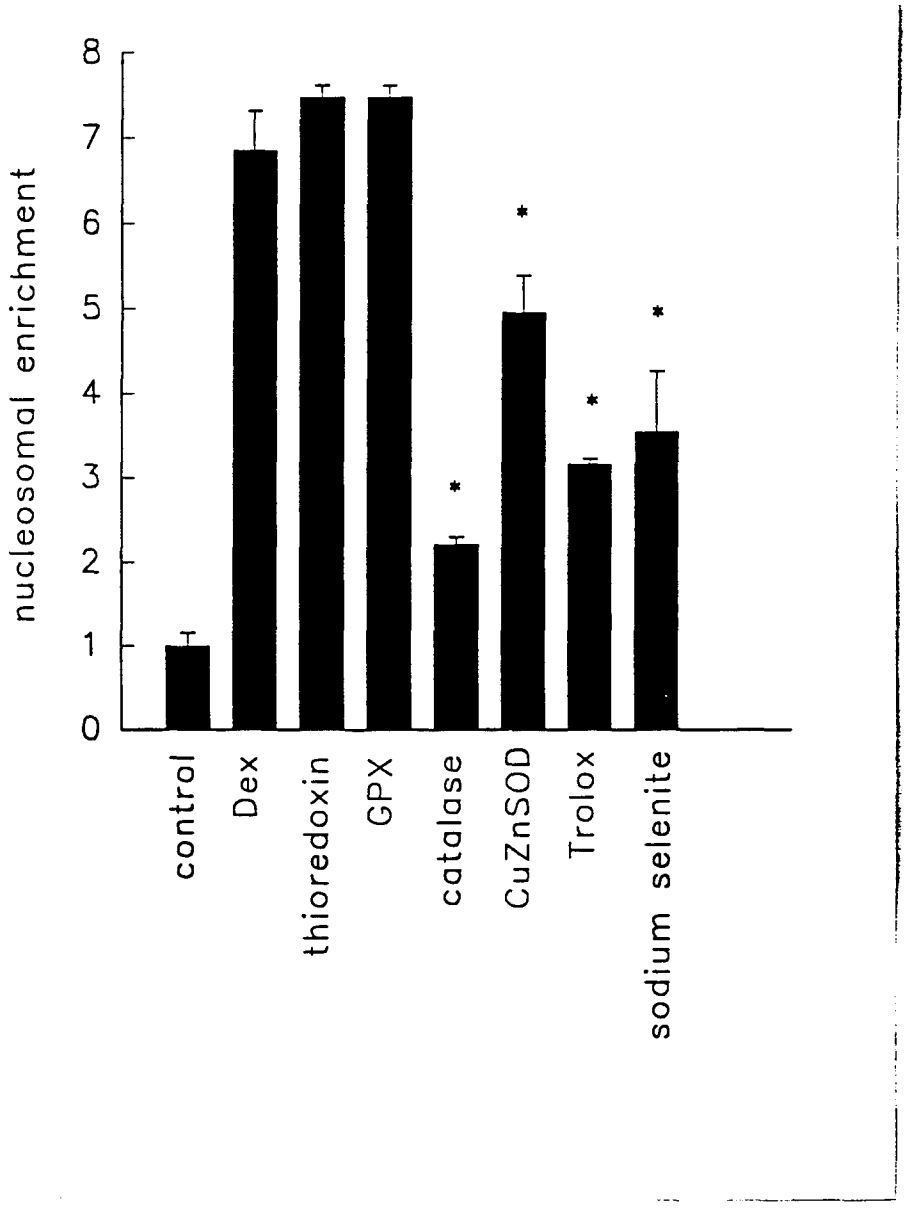
nucleosomal enrichment
0
1
2
3
4
5
6
7
8
control
Dex
thioredoxin
GPX
catalase
CuZnSOD
Trolox
sodium selenite
*
*
*
*

Figure 5

Apoptosis Journal Club: Directory of Members 8/9/95

David S. Alberts — ACC 4th Floor — 626-7685 (Phone), sharon@azcc.arizona.edu

Eileen Asher — Microbiology & Immunolgy, Immunology, AHSC 6112 — 626-1007 (Phone)

David Askew — Miesfeld Lab, BSW 519 — 626-2342 (Phone)

Amanda Baker — Pharmacology & Toxicology, Powis Lab, ACC 3955 — 626-6704 (Phone), 626-4848 (FAX)

Research: Oxidative stress in relation to apoptosis

Techniques: Enzyme assays

Carol Bernstein — Microbiology & Immunology, AHSC 6112 — 626-6069 (Phone)

Harris Bernstein — Microbiology & Immunology, AHSC 6112 — 626-6069 (Phone), 626-2100 (FAX)

Research: I collaborate with Clair Payne, Carol Bernstein, Jean Zheng, Harinder Garewal and several others on the role of bile acid and induced apoptosis in colon cancer. We are currently studying the resistance of the normal portion of the colon of colon cancer patients to induction of apoptosis - as a potential biomarker for assessing risk of colon cancer.

Publications: Payne CM, Bernstein C, and Bernstein H. Apoptosis overview emphasizing the role of oxidative stress, DNA damage and signal transduction pathways. *Leukemia and Lymphoma.* (invited article, in press).

Sanaha HS, Asher E, Payne CM, Bernstein C, and Bernstein H. Evaluation of cell death in EBV transformed lymphocytes using agarose gell electrophoresis, light microscopy and electron microscopy. *Leukemia and Lymphoma* (invited article, submitted).

Techniques: Quantification of apoptotic cells by light microscopy using Wright stained cytospin preparations.

Present Seminar: Perhaps by next summer - not now.

Margaret Briehl — Pathology, AHSC 5235 — 626-6827 (Phone), 626-1027 (FAX), mmbriehl@ccit.arizona.ed

Research: Investigating the role of oxidative stress in the mechanism of apoptosis; model systems under study are tumor necrosis-a-mediated killing of breast cancer cells and glucocorticoid-induced lymphocyte apoptosis.

Publications: Briehl MM and Miesfeld RL (1991) Isolation and characterization of transcripts induced by androgen withdrawal and apoptotic cell death in the rat ventral prostate. *Mol. Endocrin.* **5**:1381-1388

Flomerfelt FA, Briehl MM, Dowd DR, Dieken ES and Miesfeld RL (1993) Elevated glutathione S-transferase gene expression is an early event during steroid-induced lymphocyte apoptosis. *J. Cell Physiol.* **154**:573-581

Briehl MM, Cotgreave IA and Powis G (1995) Downregulation of the antioxidant defense during glucocorticoid-mediated apoptosis. *Cell Death and Differentiation* (in press)

Techniques: Detection of "DNA ladders" by agarose gel electrophoresis and end-labeling; assessing antioxidant defense status by Northern blots and enzyme assays

Present Seminar: Possibly, depending on schedule

Ihab Botros Dept. of Physiology, AHSC 4126; 626-4355 (Phone), ibotros@aruba.ccit.arizona.edu

Marianne Broome-Powell Cancer Center, ACC 3943; 626-7148 (Phone), 626-2284, mbroome@azcc.arizona.edu

Dave Clark ACC 4953; 626-2734 (Phone), 626-2445 (FAX)

Research: BCL-2 protein

Bill Dalton ACC 3945; 626-4196 (Phone), dalton@azcc.arizona.edu

Betty Dos Santos ACC 4957; 626-7753 (Phone), bdossantos@azcc.arizona.edu

Karen Duncan Phamacology & Toxicology, AHSC Pharmacy Bldg., Rm. 245; 626-5671 (Phone), duncan@tonic.pharm.arizona.edu

Wayne Edwards Dalton Lab, ACC 3933; 626-2331 (Phone), wedwards@azcc.arizona.edu

Janine Einspahr Alberts Lab, ACC 4946; 626-6865 (Phone), janine@azcc.arizona.edu

Dave Gannett Radiation Oncology, Gerner Lab, AHSC 0940; 626-5129 (Phone)

Harinder Garewal VA Medical Center; 792-1450 ext. 6600

Eugene Gerner Radiation Oncology, AHSC 0943; 626-2197 (Phone), 626-4480 (FAX), gerner@azcc.arizona.edu

Research: My lab is interested in biochemical and molecular mechanisms underlying cellular responses to stress. In this regard, we have found that a number of stresses induce polyamine acetylation. De-regulation of polyamine synthesis is also sufficient to induce apoptosis in a rat hepatoma cell line.

Publications: Harari PM, Fuller DJM, Gerner EW (1989) Heat shock stimulates polyamine oxidation by two distinct mechanisms in mammalian cell cultures. *Int. J. Radiat. Oncol. Biol. Phy.* **16**: 451-457.

Harari PM, Fuller DJM, Carper SW, Croghan MK, Meskens FL Jr, Shimm DS, Gerner EW (1990) Polyamine biosynthesis inhibitors combined with systemic hyperthermia in cancer therapy. *Int. J. Radiat. Oncol. Biol. Phy.* **19**: 89-96.

Gerner EW, Kurtts T, Fuller DJM, Casero RJ Jr (1993) Stress induction of the spermidine/spermine N^1-acetyltransferase by a post transcriptional mechanism in human and rodent cells. *Biochem. J.* **294**: 491-495.

Tome ME, Fiser SM, Gerner EW (1994) Consequences of aberrant ornithine decarboxylase regulation in rat hepatoma cells. *J. Cell. Physiol.* **158**: 237-244.

Techniques: HPLC analysis of polyamine contents
general biochemistry (analysis of glutathione, etc.)

Present Seminar: Yes

Alton (Hank) Hallum Gyn/Onc
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Lori Hazlehurst Dalton Lab
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Hana Holubec Earnest Lab
GI Department
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Pat Hoyer Physiology
AHSC 4122
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Research: Mechanisms of cell death related to luteal regression in the ovine corpus luteum as induced by prostaglandin $F_{2\alpha}$. This decision must be made at the end of the luteal phase (non-pregnant animal) or a decision to inhibit cell death must be made to maintain the corpus luteum (pregnancy). All cell types in this tissue respond within a uniform time frame.

Publications: Abstracts:
Rueda, et.al. (1993) Induction of luteal cell internucleosomal fragmentation by prostaglandin $F_{2\alpha}$ as an indicator of apoptosis. (*Biol. Reprod.* Suppl. #1, **48**:545).

Hoyer, et.al. (1994) Medium conditioned by luteolytic ovine corpora lutea inhibits endothelial cell proliferation. (Microcirc. Soc. Satellite to *FASEB* #M72

Papers:
Rueda, et.al. Internucleosomal DNA fragmentation in ovine luteal tissue associated with apoptosis: in vivo and in vitro analysis. (*Biol. Reprod.* in press)

Rueda, et.al. Medium conditioned by luteolytic ovine corpora lutea inhibits endothelial cell proliferation. (submitted for publication).

Techniques: DNA fragmentation on agarose gel electrophoresis (ethidium bromide and/or ^{32}PddATP end labeling for visualization)

in situ end labeling of DNA in tissue sections

Present Seminar: Yes

Natalia Ignatenko
Radiation Oncology
AHSC 0940
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Research: Polyamine acetylation, which increases in the plateau phase of cell growth, might relate to apoptosis.

Janet Jones
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Theresa R. Kramer
Department of Opthalmology
and Pathology
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694-4480 - 2393 (Beeper)

Hugh Laird
Pharmacology & Toxicology
AHSC Pharmacy Bldg. 234
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laird@tonic.pharm.arizona.edu

Research: Interested in metal-induced apoptotic events in cells in culture as an indicator of cytotoxicity of the metal. We are studying mercury, cadmium and zinc which are all members of a group IIb of the Periodic Table.

Techniques: We are trying to develop techniques in use by others so we are not ready to claim an "expertise" as of now!

Present Seminar: After another 6 months - yes!

Terry Landowski
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Daniel Liebler
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David Lydall
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Jesse Martinez
Radiation Oncology
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Research: Bile acid induced apoptosis ant the potential role that loss of apoptosis inducibility may have in the etiology of colon cancer.

Roger Miesfeld
Biochemistry
Bio. Sciences West 518A
626-2343 (Phone, until 1/10/95)
621-5770 (Dept. Phone, after 1/10/95)
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miesfeld@biosci.arizona.edu

Research: Molecular genetic analysis of thymocyte apoptosis. Cell line model (WEHI7.2) is being used to identify glucocorticoid-regulated early response genes and apoptosis "effector" genes.

Publications: Flomerfelt FA, Briehl MM, Dowd DR, Dieken ES, and Miesfeld R (1993) Elevated glutathione S-transferase gene expression is an early event during steroid-induced lymphocyte apoptosis. *J. Cell Phsiol.* **154**:573-581

Lam M, Dubyak G, Chen L, Nuñez G, Miesfeld R, and Distelhorst C (1994) Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca^{2+} fluxes. *Proc. Natl. Acad. Sci.* **91**:6569-6573

Flomerfelt F, and Miesfeld R (1994) Recessive mutations in a common pathway block thymocyte apoptosis induced by multiple signals. *J. Cell. Biol.* (in press)

Techniques: We have so far used standard molecular biology methods, but we would be happy to provide any support we can.

Present Seminar: Sure

Mark Nelson
Department of Pathology
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626-2619

John Nichols
Department of Opthalmology
and Pathology
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321-3665 (FAX)

Claire Payne
Microbiology & Immunology
AHSC 6112
626-2870 (Phone)
626-2100 (FAX)

Research: To understand the mechanism of apoptosis that may apply to different types of cells. To specifically understand how bile salts induce apoptosis. I will concentrate on the role of the cytoskeleton in the induction of the apoptotic process.

Publications: Payne CM and Cromey DW (1991) Ultrastructural analysis of apoptotic and normal cells using digital imaging techniques. *J. Computer-Assisted Microscopy* **3**:33-50

Payne CM, Bjore CG Jr., and Schultz DA (1992) Change in the frequencey of apoptosis after low- and high-dose X-irradiation of human lymphocytes. *J. Leukoc. Biol.* **52**:433-440

Payne CM, Bernstein C, and Bernstein H (1995) Apoptosis overview emphasizing the role of oxidative stress, DNA damage and signal transduction pathways. *Leukemia and Lymphoma* (in press)

Samaha HS, Asher E, Payne CM, Bernstein C, and Bernstein H (1995) Evaluation of cell death in EBV-transfromed lymphocytes using agarose gel electrophoresis, light microscopy and electron microscopy. I. Induction of classic apoptosis by the bile salt, sodium deoxycholate. *Leukemia and Lymphoma* (Invited article, submitted)

Techniques: morphologic assessment, includes light and electron microscopy

Present Seminar: Yes

Anthony Phillips Pediatrics, AHSC 3301, 626-5170 (Phone)

Garth Powis ACC 3rd Floor, 626-6408 (Phone)

Bo Rueda Physiology, AHSC 4126, 626-4355 (Phone), 626-2382 (FAX), brueda@aruba.ccit.arizona.edu

Research: Evaluating the expression of oxidative stress response and cell death (inducer/suppressor) genes in the ovine and bovine corpus luteum during luteolysis and pregnancy.

Publications: Rueda BR, Wegner JA, Marion SL, Wahlen DD, Hoyer PB (1994) Induction of ovine luteal internucleosomal DNA fragmentation *in vivo* and *in vitro*. *Biol. Reprod.* (in press)

Rueda BR, Hoying JB, Williams SK, Hoyer PB. Medium conditioned by luteolytic ovine corpora lutea inhibits endothelial cell proliferation (submitted)

Rueda BR, Tilly KI, Hansen TR, Hoyer PB, Tilly JL. Expression of superoxide dismutase, catalase and glutathione peroxidase in the bovine corpus luteum: Evidence supporting a role for oxidative stress in luteolysis (submitted)

Rueda BR, Tilly KI, Hansen TR, Jolly PD, Hoyer PB, Tilly JL. bcl-x, bax and p53 gene expression in the bovine corpeus luteum during structural luteolysis (submitted)

I. Glenn Sipes Pharmacology & Toxicology, AHSC Pharmacy Bldg., Rm. 238, 626-7123 (Phone)

Research: I may be directing some of my research toward this direction. As such, I have no data to present. My interests are toward the TPA induced apoptotis in prostate cancer cells.

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Quingbo Tang Dr. Josephine Lai Lab
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Research: I am studying the gene expression along the crypt-villus axis of the small intestine. The epithelial cells of villus are differentiated from the stem cells in the cryps and move up along the villus and finally die as a result of apoptosis.

Margaret Tome Biochemistry
Gerner Lab
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Research: We are studying how aberrant ornithine decarboxylase regulation induces apoptosis. Specifically, we are trying to identify the mechanism by which the resultant excess putrescine kills cells.

Dorothea Von Bredlow
Pathology
Cress Lab
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Research: My research is not directly related to apoptosis.

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David Wu Cancer Biology
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Research: I am currently interested in the p53 gene product and its effects on cells in the colon.

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Xiaozhen Xie Radiation Oncology
Gerner Lab
AHSC 0943

Breast Cancer Workshop
September 9, 1995
Arizona Cancer Center, Conference Room 2920

8:00 - 8:10 AM	Introduction	Charles Taylor, M.D. Margaret Briehl, Ph.D.
8:10 - 8:30 AM	*The AT Gene in Breast Cancer*	Garth Powis, D. Phil.
8:30 - 8:50 AM	*Understanding AT by Understanding Checkpoints in Yeast*	Ted Weinert, Ph.D.
8:50 - 9:05 AM	Discussion	
9:05 - 9:20 AM	*pH Regulation by Breast Cancer Cells in vivo and in vitro*	Natarajan Raghunand, Ph.D.
9:20 - 9:40 AM	*Role of Oxidative Stress in Apoptosis of Breast Cancer Cells*	Margaret Briehl, Ph.D.
9:40 - 9:55 AM	Discussion	
9:55 - 10:10 AM	Break	
10:10 - 10:30 AM	*Standard and Molecular Cytogenetics of Breast Cancer*	Floyd Thompson, M.S.
10:30 - 10:45 AM	Discussion	
10:45 - 11:05 AM	*Studies of Dietary Interventions for Breast Cancer Prevention*	David Alberts. M.D. Cynthia Thomson, MS, RD, CNSD
11:05 - 11:20 AM	Discussion	
11:20 - 12:00 PM	Lunch	
12:00 - 12:20 PM	*Quality of Life Research in Breast Cancer Patients*	Carrie Jo Braden, Ph.D., R.N.
12:20 - 12:35 PM	Discussion	
12:35 - 12:55 PM	*The Changing Spectrum of Mammographically Detected Early Breast Cancer*	James Warneke, M.D.
12:55 - 1:10 PM	Discussion	
1:10 - 1:30 PM	*High Dose Chemotherapy and Bone Marrow Transplantation for Breast Cancer*	Charles Taylor, M.D.
1:30 - 1:45 PM	Discussion	
1:45 - 2:05 PM	*Hyperthermia and Radiation Therapy for Breast Cancer*	Baldassarre Stea, M.D.
2:05 - 2:20 PM	Discussion	